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## CONTENTS OF VOLUME LXIV.

No. 1, MAY, 1925.


PAGE

BILLS, CHARLES E. The resistance of the antirachitic substance in cod liver oil to reagents ..... 1

CORI, CARL F., and CORI, GERTY T. The carbohydrate metabolism of tumors. I. The free sugar, lactic acid, and glycogen content of malignant tumors ..... 11

LAWSON, W. E., and SCOTT, W. O. An electrolytic modification of the Gutzeit method for the determination of arsenic in body tissues ... 23

HARINGTON, CHARLES ROBERT. Synthesis of 3, 4, 5-triiodophenylpyrrolidone carboxylic acid, a possible isomer of thyroxin ..... 29

SPEAKMAN HORACE B. The biochemistry of acetone formation from sugars by *Bacillus acetoethylicum* ..... 41

GOLDSCHMIDT, SAMUEL, and LIGHT, ARTHUR B. A method of obtaining from veins blood similar to arterial blood in gaseous content ..... 53

SANDBERG, MARTA, and BRAND, ERWIN. On papain lipase ..... 59

SANDO, CHARLES E. Anthocyanin formation in *Helianthus annuus* .... 71

MAIN, EDNA RUTH, and LOCKE, ARTHUR P. The determination of small amounts of protein nitrogen ..... 75

MORITZ, ALAN R. The effect of ultra-violet irradiation on the state of the serum calcium ..... 81

BOLLIGER, A., and HARTMAN, F. W. Observations on blood phosphates as related to carbohydrate metabolism ..... 91

HAUGE, SIGFRED M., and CARRICK, CAREY W. The antiscorbutic properties of eggs ..... 111

HOGAN, ALBERT G., GUERRANT, NOLLIE B., and KEMPSTER, HARRY L. Concerning the adequacy of synthetic diets for the growth of the chick ..... 113

LEITER, LOUIS. The metabolism of imidazoles ..... 125

GROLLMAN, ARTHUR. The combination of phenol red and proteins .. 141

MCCOLLUM, E. V., SIMMONDS, NINA, and BECKER, J. ERNESTINE. Further studies on the cause of ophthalmia in rats produced with diets containing vitamin A ..... 161

HESS, ALFRED F., and WEINSTOCK, MILDRED. The antirachitic value of irradiated cholesterol and phytosterol. II. Further evidence of change in biological activity ..... 181

HESS, ALFRED F., and WEINSTOCK, MILDRED. The antirachitic value of irradiated cholesterol and phytosterol. III. Evidence of chemical change as shown by absorption spectra ..... 193

SACKETT, GUY E. Modification of Bloor's method for the determination of cholesterol in whole blood or blood serum ..... 203




79954

XUM

BENEDICT, STANLEY R. The determination of blood sugar..... 207
BENEDICT, STANLEY R. The determination of uric acid in the blood. 215
DRUCKER, PAUL, and CULLEN, GLENN E. A simple method for obtaining cutaneous (capillary) blood from infants and adults for colorimetric pH determination..... 221

No. 2, JUNE, 1925.

SEIBERT, FLORENCE B., and LONG, ESMOND R. The interfering effect of glycerol on the biuret reaction..... 229
BAUDISCH, OSKAR, and DAVIDSON, DAVID. The mechanism of oxidation of thymine. 4, 5-Dihydroxyhydrothymine (thymine glycol). 233
JONES, D. BREESE, and GERSDORFF, C. E. F. Proteins of wheat bran. II. Distribution of nitrogen, percentages of amino acids and of free amino nitrogen: a comparison of the bran proteins with the corresponding proteins of wheat endosperm and embryo..... 241
ROSE, ANTON R. A micro method for determining nitrogen..... 253
SUMNER, JAMES B., and GRAHAM, VIOLA A. The globulins of the jack bean (*Canavalia ensiformis*). II. The content of cystine, tyrosine, and tryptophane..... 257
STEENBOCK, H., and BLACK, ARCHIE. Fat-soluble vitamins. XXIII. The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light.... 263
NELSON, E. M., and STEENBOCK, H. Fat-soluble vitamins. XXIV. The non-precipitability of the antiophthalmic and antirachitic properties from cod liver oil by digitonin..... 299
KOEHLER, A. E., BRUNQUIST, E. H., and LOEVENHART, A. S. The production of acidosis by anoxemia..... 313
ROSE, WILLIAM C., and COOK, KENNETH G. The relation of histidine and arginine to creatine and purine metabolism..... 325
ADOLPH, EDWARD F. Electrostatic forces in the diffusion of water through collodion membranes between solutions of mixed electrolytes..... 339
WU, HSIEN, and WU, DAISY YEN. Nature of heat denaturation of proteins..... 369
JACOBS, WALTER A. Saponins. III. The sapogenin occurring in *Sapindus saponaria* L. and *Sapindus mukorossi utilis* (Trabuti)... 379
JACOBS, WALTER A., and COLLINS, ARNOLD M. Strophanthin. VII. The double bond of strophanthidin..... 383
BELL, RAYMOND W. The effect of heat on the solubility of the calcium and phosphorus compounds in milk..... 391
GRIFFITH, WENDELL H. A note on the influence of the diet on the synthesis of hippuric acid..... 401
HAMMETT, FREDERICK S. A biochemical study of bone growth. I. Changes in the ash, organic matter, and water during growth (*Mus norvegicus albinus*)..... 409
SHERMAN, H. C., and MACLEOD, F. L. The calcium content of the body in relation to age, growth, and food..... 429

COLLENS, WILLIAM S. The blood supply to the pancreas with some perfusion studies ..... 461
LEVENE, P. A., and MEYER, G. M. Monoacetone galactose ..... 473
LEVENE, P. A., and ULPTS, R. On condensation of monosaccharides by means of dilute mineral acid ..... 475
COLLIP, J. B., and CLARK, E. P. Further studies on the physiological action of a parathyroid hormone ..... 485

No. 3, JULY, 1925.

HOLT, L. EMMETT, JR., LA MER, VICTOR K., and CHOWN, H. BRUCE. Studies in calcification. I. The solubility product of secondary and tertiary calcium phosphate under various conditions ..... 509
HOLT, L. EMMETT, JR., LA MER, VICTOR K., and CHOWN, H. BRUCE. Studies in calcification. II. Delayed equilibrium between the calcium phosphates and its biological significance ..... 567
HOLT, L. EMMETT, JR. Studies in calcification. III. A quantitative study of the equilibria concerned with the calcification of bone ... 579
BEZSSONOFF, NIKOLAÏ. The Jendrassik reaction for water-soluble B. Concerning Victor E. Levine's critical study ..... 589
LEVINE, VICTOR E. The Jendrassik reaction for vitamin B with reference to the work of Bezssonoff and of Levine ..... 591
DU NOÜY, P. LECOMTE. On the probable dimensions of the molecule and molecular weight of crystalline egg albumin. Plate 1 ..... 595
READ, BERNARD E. Chemical constituents of camel's urine ..... 615
DAVIDSON, DAVID, and BAUDISCH, OSKAR. The oxidation of isobarbituric acid. A new class of indigoïds ..... 619
HILLER, ALMA, LINDER, G. C., and VAN SLYKE, D. D. The reducing substances of the blood ..... 625
VOEGTLIN, CARL, THOMPSON, J. W., and DUNN, EDITH R. Hyperglycemia produced by glycerol ..... 639
STILES, H. R., PETERSON, W. H., and FRED, E. B. Fermentation products of certain mannitol-forming bacteria ..... 643
JONES, D. BREESE, GERSDORFF, C. E. F., and MOELLER, O. Proteins of the bark of the common locust tree, *Robinia pseudacacia*. I. Enzymes associated with the proteins: the composition, properties, nitrogen distribution, and some of the amino acids of the albumin. 655
JONES, D. BREESE, and CSONKA, FRANK A. Proteins of the cottonseed. 673
HAMMETT, FREDERICK S. A biochemical study of bone growth. II. Changes in the calcium, magnesium, and phosphorus of bone during growth ..... 685
HAMMETT, FREDERICK S. A biochemical study of bone growth. III. Changes in the composition of the ash during growth ..... 693
WIERZUCHOWSKI, M., and LING, S. M. Animal calorimetry. Twenty-ninth paper. On fat production in a young hog ..... 697
BAUMANN, EMIL J., and HUNT, LOUISE. On the relation of thyroid secretion to specific dynamic action ..... 709

XUM

Cullen, Glenn E., and Biilmann, Einar. The use of the quinhydrone electrode for hydrion concentration determination on serum ..... 727
Koehler, Alfred E., and Reitzel, Raymond J. The effect of pH on the oxygen consumption of tissues ..... 739
Baudisch, Oskar, and Welo, L. A. On the aging of ferrous hydroxide and ferrous carbonate ..... 753
Baudisch, Oskar, and Welo, L. A. On the aging of natural mineral waters ..... 771
Hess, Alfred F., and Helman, F. Dorothy. The phosphatide and total phosphorus content of woman's and cow's milk ..... 781
Eckstein, H. C. The fatty acids in the subcutaneous fat of man .... 797
Index to Volume LXIV ..... 807

## CORRECTION.

On page 74, Vol. lxiv, No. 1, May, 1925, in the glucose radical of the structural formulas read $C_6H_7O(OH)_4$ instead of $C_6H_7(OH)_4$.

XUM

# THE RESISTANCE OF THE ANTIRACHITIC SUBSTANCE IN COD LIVER OIL TO REAGENTS.*

By CHARLES E. BILLS.

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(Received for publication, February 24, 1925.)

The present communication records the first of a series of experiments begun at the suggestion of Professor E. V. McCollum on the chemical examination of antirachitic substances. The most familiar of these substances occurs in cod liver oil, and is commonly known as the antirachitic vitamin, or vitamin D.

The cod liver oil which I used was an unfrozen, unadulterated oil, rendered at Bay Bulls, Newfoundland, in August, 1923. 0.20 to 0.25 per cent of this oil, incorporated with the McCollum high calcium, rickets-producing diet, No. 3143, induced advanced healing in rachitic rats in 5 days. The animals were examined by the Shipley line test procedure. The samples of oil treated with reagents were similarly triturated with Diet 3143, acetone being used as diluent.

In the following records of experiments a somewhat detailed account of procedures is given. The protocols of the individual rats, however, are combined in one self-explanatory table (Table I). The rats receiving the curative preparations were kept in a *moderately lighted room.*

*Influence of Hydrogen Dioxide on the Antirachitic Vitamin.*—Mellanby (1), McCollum, Simmonds, Becker, and Shipley (2), and Goldblatt and Zilva (3) have demonstrated that hot cod liver oil does not readily lose its antirachitic activity when aerated. In the present experiment I investigated the stability of the antirachitic vitamin to hydrogen dioxide—a reagent which with

* Submitted to the Board of University Studies of the Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy.



XUM

some substances is more active than molecular oxygen. 10 cc. of oil were shaken vigorously for 5 minutes with 30 cc. of "Dioxogen." The resulting emulsion did not separate in 24 hours. The shaking was repeated daily for 18 days, and the emulsion maintained at room temperature. At the end of the 18th day the emulsion was separated centrifugally. The aqueous layer was tested for hydrogen dioxide by heating with sodium hydroxide. A vigorous ebullition of oxygen resulted.

The oil layer was observed not to have changed in smell, but the coloring matter was somewhat bleached. A portion of the oil was tested at 0.25 per cent for its antirachitic power. No considerable destruction of the vitamin was evidenced, and it may, therefore, be said that the antirachitic vitamin in cod liver oil is not destroyed by hydrogen dioxide under the conditions of this experiment.

*Influence of Hydrogen Sulfide on the Antirachitic Vitamin.*—Goldblatt and Zilva (3) and Dubin and Funk (4) have demonstrated that catalytic hydrogenation does not readily destroy the antirachitic activity of cod liver oil. My experiments indicate a similar stability toward chemical reducing agents. A shallow layer of cod liver oil was kept for 43 hours under an atmosphere of hydrogen sulfide at room temperature. The oil was then heated in an open flask for 90 minutes at 75–80°.

The oil thus treated was but little changed in odor and color. It was tested on only one rat, which received 1 per cent of the oil in his diet for 5 days. This rat, however, showed advanced healing, and the bones were exceptionally hard.

*Influence of Sulfur Dioxide on the Antirachitic Vitamin.*—Sulfur dioxide was conducted for 1 hour through 25 cc. of cod liver oil contained in a test-tube immersed in water at 65–70°. The oil was then placed in a shallow layer under a vacuum for 6 hours, while the temperature was maintained at $60 \pm 10°$.

After this treatment the oil was very dark reddish brown, and still smelled strongly of sulfur dioxide. Yet, notwithstanding its marked change in appearance, it exhibited no diminution in antirachitic activity. It is evident from the color change that sulfur dioxide reacts with some constituents of cod liver oil, but it is also evident, from the biological tests, that it does not affect the antirachitic vitamin.

XUM

*Influence of Formaldehyde on the Antirachitic Vitamin.*—With many substances formaldehyde is an active reducing agent, and there are certain organic radicles which combine with it in accordance with reactions which suggest their identity. Formaldehyde gas was conducted for 30 minutes through 15 cc. of cod liver oil contained in a test-tube immersed in water at 70°. The oil was then placed in a shallow layer under a vacuum for 6 hours, while the temperature was maintained around 95°. After this treatment the oil was little changed in color, and the odor of formaldehyde was still distinct.

The minimum dosage of this preparation was not ascertained, but large doses were found to effect complete healing. One would indeed expect so reactive a substance as formaldehyde either to react completely with the vitamin, or not at all. From the indifference of the antirachitic vitamin to formaldehyde, one may cautiously concede that it is not readily reducible; that it is not a primary amine, and probably not a secondary amine; and that it does not possess a reactive phenolic structure. The behavior of formaldehyde suggests also a means of preserving putrescible tissues for investigations in experimental rickets.

*Influence of Nitrous Fumes on the Antirachitic Vitamin.*—The oxides of nitrogen developed by the double decomposition of nitrites comprise one of the most chemically active gaseous mixtures. They react with so great a variety of chemical structures that any substance labile toward them becomes in no sense identified by its behavior; it is, however, placed in the field of familiar types, and is to this extent, characterized.

Nitrous fumes were generated by the action of 20 per cent acetic acid on sodium nitrite at room temperature. Under these conditions, if the generating flask is not agitated or shaken, a steady and gentle evolution of gas continues for many hours. The fumes were conducted for 6 hours through 40 cc. of cod liver oil contained in a test-tube immersed in water at 60–65°. The oil was then placed in a shallow layer under a vacuum for 2 hours at room temperature.

After this treatment the oil was dark reddish brown, but it remained limpid and free of precipitate. The odor was but little changed. When administered to rats in doses eight times the standard dose no antirachitic activity was evidenced.

XUM

Among the reactions of nitrous acid, the more familiar are the following: (1) deamination (primary amines); (2) formation of nitroso compounds (secondary amines); (3) the elaidin transformation (oleic acid); (4) nitration (oxybenzoic acids, pyrene, etc.); (5) oxidation (chloral, leucobases, pyrogallol, hydroquinones, methylhydrophenylacridine, etc.); (6) dehydrogenation (hydrocollidinedicarbonic acid ester, etc.); and (7) formation of addition compounds (isoprene derivatives).

*Influence of Steam on the Antirachitic Vitamin.*—In one of the commercial processes for rendering cod livers, the livers are exposed to the cooking action of direct steam. The following experiments were devised in order to ascertain whether the antirachitic vitamin is volatile with steam, and whether it is resistant to prolonged exposure to steam. They are intended to show incidentally whether the direct steam process is a permissible method of preparing cod liver oil for therapeutic purposes.

Steam from the power house was blown for 10 hours through a 2 liter Claissen flask containing 100 cc. of oil and 100 cc. of distilled water. In the 10 hours, 2,340 cc. of distillate were condensed. This was opalescent, and contained a small amount of white sediment and a few floating droplets. It exhibited a bluish cast. The odor suggested an alkylamine. On standing several hours the distillate developed minute crystals which in the sunlight appeared as bright specks of all colors. The distillate was evaporated for 42 hours at a temperature of 50–60°, the volume being reduced to about 100 cc. The odor disappeared. The concentrated distillate, with its precipitate, was incorporated with 125 gm. of Diet 3143, and dried for 16 hours at about 60°. The resulting hard cake was granulated and fed to one rat for 11 days. There was no evidence of healing.

The residuum in the Claissen flask was five times extracted with petroleum ether. By evaporating the extracts 99 cc. of the original oil were recovered. The recovered oil was somewhat intensified in color and odor. It was tested in doses as high as 2 per cent for 14 days. The antirachitic vitamin was almost completely destroyed.

The foregoing experiment was repeated with slight changes: apparatus was the same; quantity of oil and water the same; time of steaming the same; volume and appearance of distillate

the same; but instead of using steam from the power house, steam was generated by boiling *recently distilled water*.

One-half of the distillate was extracted with ethyl ether, and the other half with petroleum ether. The extracts were evaporated onto 50 gm. portions of Diet 3143, which were fed to rats for 5 days. There was no evidence of healing.

The emulsion remaining in the Claissen flask was allowed to separate by standing. Three layers resulted: an upper oil layer, a middle permanent emulsion, and a lower aqueous layer. The clear oil was tested in large doses, and its activity was found to be greatly diminished, although it remained more effective than the oil treated by steam from the power house. It was thought that the vitamin might distribute itself in the permanent emulsion after the manner of soap distributed in a froth. The oil contained in the emulsion was therefore tested, but found to be essentially the same as the oil in the upper layer. The clear aqueous layer was discarded.

From the above experiments it is evident that treatment of cod liver oil with steam slowly destroys the antirachitic vitamin. It follows that in the commercial preparation of cod liver oil the use of direct steam should not be unduly prolonged. Fortunately the period of steaming desired by most manufacturers, and required by law in Newfoundland is about 30 minutes. It is my personal observation that the livers remain at the boiling point only about half of this period, so that any damage which may theoretically occur is more than balanced by the many advantages of the direct steam process.

*Influence of Acid Hydrolysis on the Antirachitic Vitamin.*—It has been seen that steam destroys the antirachitic vitamin, presumably through hydrolysis in an approximately neutral medium. In the present experiment cod liver oil was hydrolyzed in a definitely acid medium, and both the resulting fatty acid mixture and the residual aqueous layer were tested for antirachitic activity.

1 gm. of Twitchell's reagent was triturated with 50 cc. of water. 50 cc. of oil were added and emulsified. 1 cc. of 10 per cent sulfuric acid was added, and the mixture was placed in a beaker immersed in water at 90–95° for 15 hours. At intervals it was agitated by a mechanical stirrer, and water was added to make

XUM

up for evaporation. Finally the emulsion was allowed to cool and separate by standing. Tests indicated that the hydrolysis of the fats was extensive, but not complete.

The resulting fatty acids were fed to rats in doses as high as 5 per cent. The vitamin appeared to be almost totally destroyed. The aqueous layer was administered in quantities *corresponding to* 20 per cent of the oil employed. There was no healing.

It appears, indeed, that conditions of acidification less severe than the Twitchell process suffice to destroy the antirachitic vitamin: 100 cc. of cod liver oil were emulsified with 35 cc. of 0.5 N hydrochloric acid by shaking for 5 minutes at room temperature. Without delay the emulsion was separated by rotation for 1 hour in the centrifuge. The oil layer was normal in appearance, and did not taste acid. The product when tested on a single rat at twice the normal dose gave only a faint evidence of antirachitic activity.

I desire to express my indebtedness for the guidance and facilities afforded me by Professor E. V. McCollum and Doctor P. G. Shipley. I am indebted also to Doctor Nina Simmonds for directing the selection and maintenance of all the animals used in these experiments. My thanks are extended to Mr. E. Mead Johnson, Jr., for kindly supplying the oil used in this work, and for placing at my disposal his private collection of data pertaining to its production.

## SUMMARY.

It should be borne in mind that in these experiments the nonactive constituents of cod liver oil may in some cases protect the vitamin against the reagents employed; in other cases they may accelerate its destruction.

1. The antirachitic vitamin in cod liver oil is not destroyed by hydrogen dioxide, hydrogen sulfide, sulfur dioxide, or formaldehyde.

2. It is readily destroyed by nitrous fumes, and slowly by direct steam or contact with mineral acids.

XUM

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XUM

TABLE I.

For convenience in tabulation, the quality of healing observed by the line test is here expressed on a scale of four degrees. One plus sign (+) indicates a just perceptible healing; two plus signs (++), a distinct healing; three plus signs (+++), an advanced healing; and four plus signs (++++), a practically complete healing. The reader should realize that these values bear no numerical relation to each other.

| Rat No. | Sex | Duration of treatment. | Grade of test. | Preparation administered. | Dosage in diet. | Remarks. |
|---|---|---|---|---|---|---|
| | | *days* | | | *per cent* | |
| 11 | F. | 8 | ++++ | Crude Newfoundland cod liver oil. | 0.5 | |
| 12 | M. | 8 | ++++ | " " " " " | 0.5 | |
| 13 | F. | 6 | ++++ | " " " " " | 0.5 | |
| 14 | M. | 6 | ++++ | " " " " " | 0.5 | |
| 52 | " | 5 | ++++ | " " " " " | 0.25 | |
| 105 | " | 5 | ++++ | " " " " " | 0.25 | |
| 106 | " | 5 | ++++ | " " " " " | 0.25 | |
| 50 | " | 5 | ++ | " " " " " | 0.25 | |
| 102 | " | 5 | ++++ | " " " " " | 0.20 | |
| 65 | " | 5 | +++ | " " " " " | 0.20 | |
| 101 | F. | 5 | + | " " " " " | 0.20 | |
| 34 | " | 5 | − | " " " " " | 0.20 | Doubtful. |
| 33 | M. | 5 | − | " " " " " | 0.10 | " |
| 37 | " | 5 | ++++ | Hydrogen dioxide oil. | 2 | |
| 44 | F. | 5 | +++ | " " " | 0.25 | |
| 67 | M. | 5 | ++ | " " " | 0.25 | |
| 48 | " | 5 | ++++ | " sulfide " | 1 | |
| 49 | F. | 5 | ++++ | Sulfur dioxide oil. | 5 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| 49 | F. | 5 | ++++ | Sulfur dioxide oil. | 5 | |
| 62 | M. | 5 | ++++ | " " " | 0.25 | |
| 61 | " | 5 | +++ | " " " | 0.25 | |
| 25 | " | 12 | ++++ | Formaldehyde oil. | 2 | |
| 26 | F. | 7 | ++++ | " " | 2 | |
| 1 | M. | 14 | − | Nitrous fumes oil. | 2 | |
| 2 | F. | 14 | − | " " " | 2 | |
| 19 | " | 11 | − | First steam distillate, total, evaporated. | (?) | |
| 23 | M. | 14 | + | " " treated oil residue. | 2 | Doubtful. |
| 24 | " | 14 | − | " " " " " | 2 | |
| 72 | " | 5 | − | " " " " " | 2 | |
| 20 | F. | 5 | − | " " " " " | 1 | |
| 80 | M. | 5 | − | Second steam distillate, ethyl ether extract. | (?) | |
| 87 | " | 5 | − | Same, but extracted with petroleum ether. | (?) | |
| 83 | " | 5 | ++ | Second steam treated oil residue. | 5 | |
| 84 | " | 5 | + | " " " " " | 5 | |
| 85 | " | 5 | +++ | " " " " " | 2 | |
| 86 | " | 5 | ++ | " " " " " | 2 | |
| 81 | F. | 5 | +++ | Oil in permanent emulsion after steaming. | 3 | |
| 82 | M. | 5 | − | " " " " " " | 3 | |
| 15 | " | 14 | − | Twitchell process fatty acids. | 2 | |
| 16 | " | 8 | − | " " " " | 2 | |
| 57 | " | 5 | − | " " " " | 2 | |
| 58 | " | 5 | − | " " " " | 2 | |
| 79 | " | 5 | + | " " " " | 5 | |
| 17 | " | 14 | − | " " aqueous layer. | "20" | |
| 18 | F. | 8 | − | " " " " | "20" | |
| 27 | " | 5 | + | Hydrochloric acid oil residue. | 0.5 | |

XUM

# THE CARBOHYDRATE METABOLISM OF TUMORS.

## I. THE FREE SUGAR, LACTIC ACID, AND GLYCOGEN CONTENT OF MALIGNANT TUMORS.

By CARL F. CORI and GERTY T. CORI.

(*From the State Institute for the Study of Malignant Disease, Buffalo.*)

(Received for publication, February 4, 1925.)

Warburg, Posener, and Negelein (1) discovered the important fact that surviving tumor tissue showed an unusually large glycolytic power when compared with other tissues under identical experimental conditions. All other tissues including embryonic tissue showed some glycolysis in the absence of oxygen, but practically none when oxygen was present, while the glycolytic power of tumor cells was only slightly decreased by the presence of oxygen. This unique behavior of tumor tissue among all other tissue, namely to produce more lactic acid from glucose than can be removed by oxidation, induced Warburg to compare the metabolism of the tumor cells with that of fermenting yeast. Warburg also adduced evidence that the glycolysis was bound to the structure and did not take place in the liquid medium of the cells, which made it very probable that the energy derived from the splitting of glucose into lactic acid was utilized by the tumor cells; in other words, that the phenomena observed *in vitro* were also taking place *in vivo*. The experiments reported in this paper bring forward some evidence that tumor tissue in the living animal splits large amounts of glucose into lactic acid. Our first experiments dealt with the free sugar content of tumors under various conditions and it was only after Warburg's work came to our notice that lactic acid determinations were included.

### EXPERIMENTAL.

#### *1. Methods.*

The analyses were performed on spontaneous mammary carcinoma of the mouse, on two types of transplantable mouse car-



XUM

cinoma, on Jensen rat sarcoma, and on two human tumors. Many tumors had to be discarded on account of necrosis or cystic degeneration. As a rule only such tumors were used for analysis as were entirely solid and of even white appearance upon a macroscopic examination. Even small necroses were easily detected by their yellowish tinge and by their soft consistency. Microscopic examination of these tumors showed that they were mainly composed of tumor cells and that connective tissue formed only a small percentage of the tumor mass.

The free sugar (2), lactic acid (3), and total carbohydrate (4) content of the tumors were determined by methods which have been carefully studied on other tissues and which have been described previously. The free sugar was extracted from the macerated tumors with boiling water, the proteins were precipitated with colloidal iron, and the sugar, after removal of interfering substances with Lloyd's reagent, was determined by the Hagedorn and Jensen (5) method. The lactic acid was determined in the filtrate from Schenk's precipitation after removing mercury by $H_2S$ and the glucose and other interfering substances by the Salkowski-Van Slyke procedure. The lactic acid in the final filtrate was then determined by the Clausen (6) $H_2SO_4$ method. In some instances the same Schenk filtrate that was used for the lactic acid determination was also analyzed for inorganic phosphates by the Briggs (7) modification of the Bell and Doisy method. If a separate piece of the tumor was analyzed for inorganic phosphates, it was treated exactly as for the lactic acid determination with the exception of the Salkowski-Van Slyke precipitation. Organic phosphates of the so called lactacidogen fraction and the lactic acid maximum were obtained by incubating macerated tumor tissue with 1 per cent $NaHCO_3$ for 2 hours at 37°C. Glycogen was determined by Pflueger's (8) simplified method. For blood sugar Hagedorn and Jensen's (5) procedure was used.

### *2. Free Sugar in Tumor Tissue.*

It was found previously (2) that the water used for the extraction of the tissue sugar dissolves also a number of other substances, as uric acid, creatinine, and creatine, which have reducing properties. These interfering substances, which were responsible for

the "rest reduction" could easily be removed by shaking out with Lloyd's reagent. There was a considerable "rest reduction" in water extracts of muscle, kidney, and brain, while none was found in water extracts of the liver. Tumor tissue also shows a high "rest reduction" as can be seen from Table I.

Table II summarizes the values for the free sugar content of malignant tumors, which were obtained at a normal or slightly increased blood sugar level of the tumor-bearing animals. It should be noted that there is no marked difference in the free sugar content of carcinoma on the one hand and sarcoma on the other hand. Table III, in which a comparison is made between the free

TABLE I.

*Effect of Shaking Out with Lloyd's Reagent on the Reducing Power of Water Extracts of Tumor Tissue.*

| Free sugar in tumor. | |
|---|---|
| Before treatment with Lloyd's reagent. | After treatment with Lloyd's reagent. |
| *mg. per 100 gm.* | *mg. per 100 gm.* |
| 87 | 55 |
| 87 | 54 |
| 84 | 52 |
| 82 | 51 |
| 69 | 46 |
| 58 | 36 |
| Average.....77.8 | 49 |

Diminution in reducing power by treatment with Lloyd's reagent: 37 per cent.

sugar content of tumor tissue with that of other tissues of the mouse, shows that the lowest free sugar content is found in the tumors. Since this applies to tumors of different histological origin, namely to carcinoma and to sarcoma, it seems very probable that a very low free sugar content is characteristic of malignant tumors in general.

In view of the low free sugar content of tumors at a normal blood sugar level it seemed of interest to study the permeability of tumor tissue for glucose at blood sugar levels higher and lower than normal. An increase in blood sugar was produced by the administration of glucose or by epinephrine while the blood sugar

XUM

TABLE II.

*Normal Free Sugar Content of Mouse Carcinoma and Jensen Rat Sarcoma.*

| Length of time of fasting. | Weight of tumor. | Blood sugar. | Free sugar in tumor. |
|---|---|---|---|
| *hrs.* | *gm.* | *per cent* | *per cent* |
| 0 | 2.73 | | 0.042* |
| 0 | 3.73 | | 0.053† |
| 1 | 2.56 | | 0.050† |
| 1 | 1.50 | | 0.044* |
| 3 | 1.04 | 0.166 | 0.051† |
| 3 | 0.64 | 0.175 | 0.053† |
| 3 | 1.58 | 0.130 | 0.046‡ |
| 3 | 1.37 | 0.173 | 0.054‡ |
| 4 | 1.78 | 0.126 | 0.052† |
| 14 | 3.40 | | 0.039* |
| 15 | 0.97 | 0.127 | 0.055† |
| 15 | 1.45 | 0.117 | 0.036† |
| 17 | 0.68 | 0.110 | 0.059† |
| 17 | 0.22 | 0.130 | 0.047† |
| 20 | 0.71 | 0.105 | 0.061† |
| 24 | 0.25 | 0.126 | 0.069‡ |
| Average..... | | 0.135 | 0.051 |

* Jensen rat sarcoma.
† Spontaneous mouse carcinoma.
‡ Transplanted mouse carcinoma.

TABLE III.

*Comparison of the Free Sugar Content of Jensen Rat Sarcoma and of Mouse Carcinoma with the Free Sugar Content of Other Tissues of the Mouse.*

| Type of tissue. | Free sugar content. | Average length of time of fasting. | Remarks. |
|---|---|---|---|
| | *per cent* | *hrs.* | |
| Jensen rat sarcoma..... | 0.041 | 1 | Average of 3 experiments. |
| Mouse carcinoma..... | 0.053 | 10 | " " 13 " |
| Liver*..... | 0.357 | 1 | " " 15 " |
| | 0.198 | 19 | " " 10 " |
| Muscle*..... | 0.079 | 20 | " " 8 " |
| Kidney*..... | 0.126 | 19 | " " 10 " |
| Brain*..... | 0.059 | 18 | " " 4 " |

* Calculated from previous experiments (2).

XUM

was lowered by insulin. Table IV contains the data on the effect of glucose and Table V on the influence of epinephrine administration. It will be noted that with increasing blood sugar concen-

TABLE IV.

*Effect of Glucose Administration on the Free Sugar Concentration of the Tumors.*

100 mg. of glucose were given intraperitoneally to mice fasted previously for 17 to 24 hours and weighing from 18 to 25 gm. The tumors were analyzed 15, 30, 60, 90, and 120 minutes after the glucose injection.

| 15 min. | | 30 min. | | 60 min. | | 90 min. | | 120 min. | |
|---|---|---|---|---|---|---|---|---|---|
| Blood sugar. | Free sugar in tumor. | Blood sugar. | Free sugar in tumor. | Blood sugar. | Free sugar in tumor. | Blood sugar. | Free sugar in tumor. | Blood sugar. | Free sugar in tumor. |
| *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 0.396 | 0.195 | 0.230 | 0.260 | 0.166 | 0.057 | 0.144 | 0.070 | 0.124 | 0.060 |
| 0.527 | 0.176 | 0.382 | 0.262 | 0.176 | 0.060 | | | | |
| 0.607 | 0.287 | 0.392 | 0.311 | 0.186 | 0.078 | | | | |
| 0.634 | 0.255 | 0.464 | 0.184 | 0.194 | 0.076 | | | | |
| Average.....0.541 | 0.228 | 0.367 | 0.254 | 0.180 | 0.068 | | | | |

TABLE V.

*Effect of Epinephrine on the Free Sugar Concentration of the Tumors.*

0.05 mg. of epinephrine was injected subcutaneously and the tumors were analyzed 15, 30, 60, and 150 minutes later.

| 15 min. | | 30 min. | | 60 min. | | 150 min. | |
|---|---|---|---|---|---|---|---|
| Blood sugar. | Free sugar in tumor. | Blood sugar. | Free sugar in tumor. | Blood sugar. | Free sugar in tumor. | Blood sugar. | Free sugar in tumor. |
| *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 0.198 | 0.103 | 0.276 | 0.065 | 0.258 | 0.062 | 0.166 | 0.057 |
| 0.276 | 0.096 | 0.282 | 0.094 | 0.174 | 0.063 | | |
| 0.298 | 0.100 | 0.288 | 0.081 | | | | |
| 0.312 | 0.159 | 0.294 | 0.090 | | | | |
| Average.....0.271 | 0.114 | 0.285 | 0.082 | | | | |

tration the free sugar content of the tumors was also increased. Thus 15 minutes after the glucose injection the free sugar content of the tumors was raised four and one-half times, 30 minutes after

XUM

the injection five times, while 60 and 90 minutes after the glucose injection with the return of the blood sugar to normal, the free sugar concentration of the tumors also approached the normal. These results show that tumor tissue is very easily permeable to glucose at higher blood sugar levels. The determination of the free sugar concentration is, of course, not a quantitative measure, but only a rough estimate of permeability, since simultaneously with the intake of glucose by the tissues, there is also a removal of glucose, the magnitude of which is not taken into account. A lowering of the blood sugar level by insulin (Table VI) caused only a very slight diminution of the free sugar content of the tumors.

TABLE VI.

*Influence of Insulin on the Free Sugar Content of Tumors.*

0.16 unit of insulin was injected intraperitoneally.

| Blood sugar. | Free sugar in tumor. |
|---|---|
| *per cent* | *per cent* |
| 0.016 | 0.029 |
| 0.032 | 0.059 |
| 0.037 | 0.056 |
| 0.075 | 0.038 |
| Average.....0.040 | 0.045 |

### *3. Lactic Acid in Tumors.*

The work of Warburg made it desirable to obtain accurate data on the lactic acid content of tumors under normal conditions. Only a limited number of analyses could be performed, since it was difficult to obtain tumors of a sufficiently large size which were entirely free from necrosis or cysts.

From the experiments of Warburg it may be calculated that tumor cells in the presence of oxygen, in a Ringer solution containing 0.04 per cent glucose, form in 1 hour an amount of lactic acid equivalent to about 1 per cent of their fresh weight. Yet the results of Table VII and the comparison of the lactic acid values in Table VIII indicate clearly that the tumors have a very low lactic acid content under normal conditions. This was rather surprising and there seemed to be only two explanations possible. Either the strong glycolytic power of tumor cells

XUM

described by Warburg was only an *in vitro* reaction due to the more or less abnormal conditions to which the tumor cells were subjected, or, if the glycolysis was of the same magnitude *in vivo* as *in vitro* the lactic acid was carried away by the blood

TABLE VII.

*Normal Lactic Acid Content of Mouse Carcinoma and Jensen Rat Sarcoma.*

| Length of time of fasting. | Weight of tumor. | Free sugar in tumor. | Lactic acid in tumor. |
|---|---|---|---|
| *hrs.* | *gm.* | *per cent* | *per cent* |
| 0 | 1.20 | | 0.022* |
| 0 | 3.72 | 0.053 | 0.041† |
| 0 | 1.61 | | 0.060* |
| 1 | 2.56 | 0.050 | 0.013* |
| 1 | 0.78 | | 0.015* |
| 2 | 1.33 | | 0.022* |
| 2 | 1.72 | | 0.054* |
| 14 | 3.74 | 0.040 | 0.048† |
| 17 | 1.93 | 0.038 | 0.036‡ |
| Average..... | | | 0.034 |

* Spontaneous mouse carcinoma.

† Jensen rat sarcoma.

‡ Metastatic mammary carcinoma (human).

TABLE VIII.

*Comparison of the Lactic Acid Content of Mouse Carcinoma and Jensen Rat Sarcoma with the Lactic Acid Content of Other Tissues of the Mouse.*

| Type of tissue. | Lactic acid content. | Length of time of fasting. | Remarks. |
|---|---|---|---|
| | *per cent* | *hrs.* | |
| Mouse carcinoma..... | 0.031 | 0–2 | Average of 6 experiments. |
| Jensen rat sarcoma..... | 0.044 | 0–14 | " " 2 " |
| Liver*..... | 0.051 | 0–2 | " " 15 " |
| | 0.011 | 17–22 | " " 10 " |
| Muscle*..... | 0.112 | 1–28 | " " 16 " |

* Calculated from previous experiments (3).

stream as fast as it was formed. Warburg found that the rate of glycolysis of surviving tumor tissue was increased with increasing glucose concentration. Thus raising the sugar concentration from 0.04 to 0.2 per cent nearly doubled the rate of

XUM

glycolysis, while just above 0.2 per cent glucose the rate reached its maximum. Since the normal free sugar concentration of the tumors was, as an average, only 0.051 per cent (Table II) it seemed possible that the rate of glycolysis was not sufficiently large to produce an accumulation of lactic acid in the tumors. The fact that the free sugar content of the tumors could be raised to 0.2 per cent by the administration of glucose (Table IV) made it possible to increase the lactic acid production in the tumors to such an extent that an accumulation of lactic acid occurred. This is illustrated in Table IX. After glucose administration the lactic acid content of the tumors was four times higher than normal. In contrast to this behavior of tumor tissue the lactic acid content of the liver was not altered by glucose injections (Table X). Such a high lactic acid content of the tumors should lead to an increase in the lactic acid concentration of the systemic blood, provided the tumor constitutes a sufficiently large percentage of the body weight. Our preliminary data recorded in Experiments 1 to 7 (Table IX) seem to point in this direction. Thus glucose administration raised the blood lactic acid of the tumor-bearing animals to 0.080 and 0.075 per cent respectively, while seven suitable control animals showed lactic acid values ranging from 0.017 to 0.034 per cent. The values for the inorganic and organic phosphorus in tumors, recorded in Experiments 1 to 7, Table IX, are also of a preliminary character, since they are not numerous enough to warrant definite conclusions.

TABLE IX.

*Effect of Glucose Administration on the Lactic Acid Concentration in Tumors.*

| Experiment No. | Blood sugar. | Free sugar in tumor. | Lactic acid in tumor. |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| 1 | 0.224 | 0.164 | 0.117 |
| 2 | 0.480 | 0.255 | 0.160 |
| 3 | | 0.259 | 0.166 |
| 4 | 0.366 | 0.183 | 0.141 |
| 5 | | 0.110 | 0.098 |
| 6 | | 0.215 | 0.096 |
| 7 | 0.166 | 0.145 | 0.179 |
| Average | | 0.190 | 0.137 |

XUM

*Experiment 1.*—Rat with a very large sarcoma. Weight of rat 234.5 gm. Weight of tumor 32 gm. or 13.7 per cent of the body weight. 2.37 gm. of glucose were given by stomach tube and the rat was killed 1½ hours later. The blood contained 0.085 per cent of lactic acid. The tumor contained 0.265 per cent of glycogen, 0.029 per cent of inorganic phosphates, and 0.154 per cent of organic phosphates (lactacidogen fraction) and gave a lactic acid maximum (in 1 per cent $NaHCO_3$) of 0.184 per cent.

*Experiment 2.*—Mouse with very large spontaneous tumor. Weight of mouse 39.6 gm. Weight of tumor 8.74 gm. or 22 per cent of the body weight. 400 mg. of glucose were given intraperitoneally and the mouse was killed 1½ hours later. The blood contained 0.075 per cent of lactic acid. The tumor contained 0.658 per cent of total carbohydrates, 0.033 per cent of inorganic phosphates, and 0.151 per cent of organic phosphates (lactacidogen fraction) and gave a lactic acid maximum (in 1 per cent $NaHCO_3$) of 0.382 per cent.

*Experiment 3.*—Mouse with spontaneous tumor. Weight of mouse 29.1 gm. Weight of tumor 1.3 gm. or 4.46 per cent of the body weight. 200 mg. of glucose were given intraperitoneally and the mouse was killed after 45 minutes.

*Experiment 4.*—Mouse with spontaneous tumor. Weight of mouse 30.3 gm. Weight of tumor 1.1 gm. or 3.67 per cent of the body weight. 140 mg. of glucose were injected intraperitoneally and the mouse was killed after 1 hour.

*Experiment 5.*—Mouse with spontaneous tumor. Weight of tumor 1.23 gm. Injected with 100 mg. of glucose and killed after 1 hour.

*Experiment 6.*—Mouse with spontaneous tumor. Weight of mouse 32.1 gm. Weight of tumor 2.2 gm. or 6.8 per cent of the body weight. 200 mg. of glucose were injected subcutaneously and the mouse was killed after 30 minutes.

*Experiment 7.*—Patient with a metastasizing melano-carcinoma. Blood sugar before, 0.127 per cent. Blood lactic acid before, 0.022 per cent. 100 gm. of glucose were given *per os*. 1 hour later blood sugar 0.166 per cent, blood lactic acid 0.039 per cent. 2 hours after the glucose ingestion a metastatic growth, located in the axilla, was removed under local anesthesia and glycolysis immediately checked by freezing with compressed $CO_2$. The tumor contained 0.852 per cent of total carbohydrates, 0.033 per cent of inorganic phosphates, and 0.156 per cent of organic phosphates (lactacidogen fraction) and gave a lactic acid maximum (in 1 per cent $NaHCO_3$) of 0.419 per cent.

### *4. Glycogen in Tumors.*

That malignant tumors contain considerable amounts of glycogen has long been known, especially through the microchemical methods of pathologists. Our data show likewise that this reserve carbohydrate is abundantly present in tumors. Glucose ingestion

XUM

seems to increase the glycogen content of the tumors as can be judged from the glycogen and lactic acid maximum values given in Experiments 1 to 7, Table IX.

TABLE X.

*Effect of Glucose Administration on the Lactic Acid Content of the Mouse Liver.*

The mice were fasted from 1 to 3 hours prior to the experiments. 100 mg. of glucose were given intraperitoneally and the mice killed 1 hour after the injection.

| Free sugar in liver. | Lactic acid in liver. |
|---|---|
| *per cent* | *per cent* |
| 0.391 | 0.060 |
| 0.310 | 0.038 |
| 0.282 | 0.034 |
| 0.300 | 0.070 |
| | 0.041 |
| Average | 0.048 |

Average for control mice 0.051 (see Table VIII).

TABLE XI.

*Glycogen Content of Mouse Carcinoma and Jensen Rat Sarcoma.*

| Length of time of fasting. | Weight of tumor. | Blood sugar. | Glycogen in tumor. |
|---|---|---|---|
| *hrs.* | *gm.* | *per cent* | *per cent* |
| 1 | 2.94 | 0.126 | 0.176* |
| 1 | 1.87 | 0.141 | 0.183* |
| 2 | 6.14 | 0.152 | 0.223* |
| 4 | 3.62 | 0.149 | 0.171* |
| 4 | 6.27 | 0.168 | 0.303* |
| 14 | 23.5 | 0.094 | 0.122† |
| Average | | | 0.196 |

* Spontaneous mouse carcinoma.

† Jensen rat sarcoma.

DISCUSSION.

It seems very difficult to demonstrate an increased lactic acid production in the tissues of the living animal, since up to a cer-

tain limit an excess of lactic acid that is formed in the tissues is eliminated into the blood stream. Some evidence for this was found in former experiments (3). During insulin convulsions or after epinephrine injections a strong increase in the lactic acid content of the blood was noted, while muscles and liver, which were undoubtedly the source of this increase, showed a normal lactic acid content. For these reasons the low lactic acid content of tumors of fasting animals must not be taken as a contradiction of Warburg's experiments. It was shown that after glucose administration the lactic acid content of the tumor tissue was strongly increased. The reason for this seems to be that the rate of glycolysis of the tumor tissue in the living animal is dependent on the free sugar concentration. Tumors of fasting animals showed a low free sugar concentration. After glucose injection the free sugar concentration was strongly increased and this resulted in such an excess of lactic acid production that the lactic acid could not completely be carried away by the blood stream. These findings in the living animal were considered to be analogous to Warburg's *in vitro* experiments in which the rate of glycolysis of surviving tumor tissue was increased with increasing glucose concentration. Our experiments bring forward some evidence that the important results of Warburg on the metabolism of surviving tumor tissue are also valid for *in vivo* conditions.

SUMMARY.

1. Spontaneous and transplanted mouse carcinoma and Jensen rat sarcoma showed at a normal blood sugar level of the tumor-bearing animals a free sugar content of 0.051 per cent (average of 16 experiments) and a lactic acid content of 0.034 per cent (average of 9 experiments).

2. After glucose administration the free sugar concentration of these tumors rose to 0.228 and 0.254 per cent (average of 4 experiments each) and the lactic acid concentration to 0.137 per cent (average of 7 experiments).

3. The glycogen content of six individual tumors was as an average 0.196 per cent.

XUM

We wish to thank Dr. Woglom of the Cancer Institute of Columbia University for supplying us with the transplantable Jensen rat sarcoma and Mr. Marsh of this Institute for the mice with spontaneous tumors.

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XUM

# AN ELECTROLYTIC MODIFICATION OF THE GUTZEIT METHOD FOR THE DETERMINATION OF ARSENIC IN BODY TISSUES.*

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In the biochemical laboratory at Edgewood Arsenal there have always been objections to the Gutzeit method as described by Klein (1) although the method has been used, with some slight variations, for a number of years. The objections to the method are based on no fault or error in the procedure recommended by Klein, which is undoubtedly the best yet submitted, but because of special difficulties encountered in the analysis of different biological tissues. The digestion of organs or muscle is difficult, requiring varying amounts of sulfuric acid for different samples, thereby giving final solutions for testing that contain different volumes of acid. The zinc used in these experiments for the formation of hydrogen must be carefully purified, even that sold as arsenic-free being found to give slight stains on the mercuric bromide paper. On account of the varying amounts of acid present, the determinations must be carefully watched through the entire runs, because the speed of generation of hydrogen may change measurably the length of the stain. As the amounts of arsenic usually encountered in the tissue are slight, usually from 0.001 to 0.015 mg. of arsenious oxide per gm. of tissue and seldom more than 0.020 mg., variations which may be considered of minor importance in other cases are of great importance in our work. On account of the small amounts of arsenic in the organs, titration with standard solutions, as recommended by different investigators (2, 3, 4), is out of the question.

* Published with the permission of the Chief of the Chemical Warfare Service.



XUM

An electrolytic method for the determinations of arsenic as arsine is mentioned in Treadwell-Hall's Analytical Chemistry (5), using the apparatus devised by Thorpe (6) which employs a current of 2 to 3 amperes with lead-platinum electrodes in a sulfuric acid concentration of 1 to 7. The use of a lead cathode prevents any retention of arsenic.

When this method was tried by us, using the acidity and current density recommended, the formation of hydrogen sulfide was so great that the arsenic stains were entirely obliterated. By varying the acidity and amount of current we were finally able to reduce the formation of hydrogen sulfide to a negligible factor and still have complete decomposition of the arsenic into arsine as shown by blank runs after the completion of the determinations.

*Apparatus.*

The apparatus as finally developed is shown in Fig. 1, which is self-explanatory.

A current of 0.9 ampere at 5 volts was used. In our work two of these cells were connected in series permitting two determinations at the same time. The cathode is of pure sheet lead, rolled into a tube around a $\frac{5}{8}$ inch test-tube. The anode is a platinum foil, measuring 1 by $2\frac{1}{2}$ inches and weighing 4 gm.

The lead acetate paper is prepared by cutting ordinary filter paper into strips, rolling them into a spiral, and slipping into the tube above the glass wool. After closing the upper end of the tube with a cotton plug, a sufficient amount of 2 per cent lead acetate solution to moisten the paper is poured in, and the water evaporated by drying in a vacuum oven.

The preparation of mercuric bromide paper requires special care to secure even impregnation. The filter paper which was found to give the best results was Eimer and Amend's white No. 10 of the common bibulous type, furnished in sheets 15 by 18 inches in size. A sheet is cut up into smaller ones of one-quarter the size and marked by a line across one end to designate the end by which the paper is suspended in drying. After soaking in a 5 per cent solution of mercuric bromide in 75 per cent alcohol it is hung in a dark room to dry. When dry about an inch of the lower edge is cut off, and the remainder is cut into strips of exactly 2

and 3 mm. width. The end opposite the pencil mark is always the one inserted into the Gutzeit tube. The strips of paper are

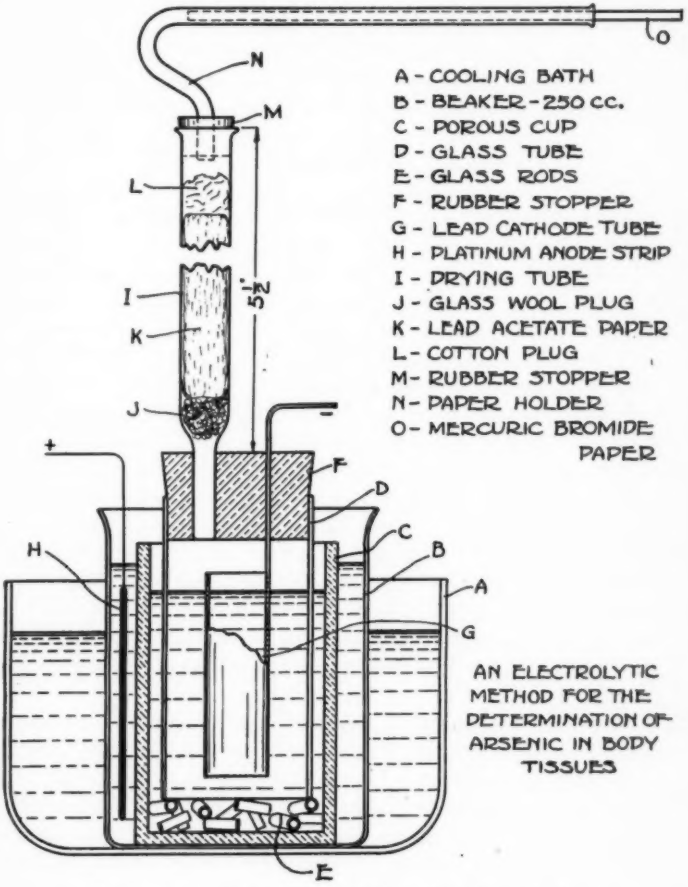
A - COOLING BATH
B - BEAKER - 250 cc.
C - POROUS CUP
D - GLASS TUBE
E - GLASS RODS
F - RUBBER STOPPER
G - LEAD CATHODE TUBE
H - PLATINUM ANODE STRIP
I - DRYING TUBE
J - GLASS WOOL PLUG
K - LEAD ACETATE PAPER
L - COTTON PLUG
M - RUBBER STOPPER
N - PAPER HOLDER
O - MERCURIC BROMIDE PAPER
AN ELECTROLYTIC METHOD FOR THE DETERMINATION OF ARSENIC IN BODY TISSUES
5 cm.
+
-

Fig. 1. Apparatus for electrolytic Gutzeit method.

best preserved in a stoppered test-tube. The use of phosphorus pentoxide or other drying agent is not recommended, as a trace of moisture in the paper is essential for the formation of sharp stains.

The sulfuric acid used for the digestion of the samples must not contain over 0.0000025 per cent of arsenic. If it contains more than this it should be purified, preferably by treatment with hydrochloric acid and sulfur dioxide and heating.

The stannous chloride solution contains 80 gm. of stannous chloride in 100 cc. of water to which has been added 5 cc. of arsenic-free hydrochloric acid.

*Procedure.*

10 gm. of the tissue to be tested are chopped fine and placed in a 500 cc. Pyrex Kjeldahl flask. 20 cc. of arsenic-free sulfuric acid are added, about 5 gm. of potassium sulfate, and a crystal of copper sulfate. The flask and contents are heated cautiously at first until the material no longer foams, then more strongly until the solution is colorless. Occasionally more sulfuric acid must be added. The solution is cooled and diluted in a volumetric flask to 100 cc. and 20 cc. are pipetted into a 100 cc. Erlenmeyer flask. To this is added from 0.5 to 1 cc. of stannous chloride solution and the mixture heated to boiling. After cooling the flask, the contents are diluted to 50 cc. and poured into the porous cup. 75 cc. of 4 per cent sulfuric acid are pipetted into the beaker outside of the porous cup, the stopper is replaced, and the solution is electrolyzed with a current of 0.9 ampere at 5 volts, over a period of 1 hour. The length of stain on the mercuric bromide paper is compared with standards made with pure arsenious oxide, and the amount of arsenic per gm. determined from the aliquot part taken for analysis.

Standards are made by using different volumes of arsenious oxide solution, containing definite amounts of the trioxide, ranging from 0.001 to 0.015 mg. For these standards mercuric bromide paper of 2 mm. width is used. For larger amounts, ranging from 0.010 to 0.060 mg., the paper is cut 3 mm. in width. Paper holders of different sizes must be used for the two widths of paper to avoid loss of arsenic. The standards may be preserved under glass, and if kept in the dark when not in use will last for a considerable length of time. If desired, advantage may be taken of the fact that the upper limits of the stains for correspondingly increasing amounts of arsenic lie on a straight line. By deter-

XUM

mining the slope of this line, as suggested by Klein (1), the use of standards may be omitted, only the straight line curve being necessary.

## DISCUSSION.

The use of stannous chloride solution is essential in order to make certain of reducing all the arsenic to the trivalent form.

TABLE I.

| Tissue. | $As_2O_3$ added per gm. tissue. | $As_2O_3$ added per gm. tissue ($As_2O_3$ equivalent). | $As_2O_3$ recovered. | |
|---|---|---|---|---|
| | | | No $SnCl_2$ used. | $SnCl_2$ used. |
| | *mg.* | *mg.* | *mg.* | *mg.* |
| Kidney | 0.002 | | 0.0015 | 0.002 |
| Heart | 0.002 | | 0.002 | |
| Pancreas | 0.002 | | 0.0005 | 0.002 |
| | 0.004 | | 0.001 | 0.004 |
| Spleen | 0.002 | | 0.0015 | 0.002 |
| Kidney | 0.004 | | 0.002 | 0.003 |
| Liver | 0.004 | | 0.001 | 0.004 |
| | 0.004 | | | 0.004 |
| | 0.005 | | | 0.005 |
| | 0.006 | | | 0.006 |
| | 0.007 | | | 0.007 |
| | 0.008 | | | 0.008 |
| | | 0.002 | 0.000 | 0.002 |
| | | 0.002 | | 0.002 |
| Aqueous solution, no tissue | | 0.004 | | 0.004 |
| | | 0.005 | | 0.005 |
| | 0.004 | | | 0.004 |
| | 0.012 | | | 0.012 |
| | 0.003 | | | 0.003 |
| | 0.009 | | | 0.008 |

Sulfurous acid was tried, but the reduction was often incomplete, even after boiling. Potassium iodide offered no advantages over stannous chloride and, with us, gave varying results on the pentavalent salts. Our results when using not more than 1 cc. of stannous chloride solution were practically always accurate, full reduction of the pentavalent salts being secured. The addition of ferrous chloride recommended for the zinc modification of the Gutzeit method was found to have no influence on the

length or intensity of the stain with the electrolytic method and was not continued.

In addition to the large number of tests run with standard arsenious oxide solution by which the feasibility of the method was determined, experiments were also carried out on the destruction and analysis of organs to which definite amounts of arsenic trioxide or pentoxide had been added.

Table I shows the accuracy obtained when following the procedure and using the apparatus recommended.

The results secured when using stannous chloride solution always accounted for all the arsenic added, whether it was added in the trivalent or pentavalent state.

SUMMARY.

1. Modifications in the electrolytic Gutzeit method for determining arsenic are proposed, which will do away with certain objections to the zinc-acid method of producing hydrogen for this determination.

2. The addition of stannous chloride to the solution is required in order to reduce all the arsenic to the trivalent form. Oxidation of part of the arsenic takes place when the tissue is destroyed with sulfuric acid and arsenic salts are not reduced to arsine by nascent hydrogen.

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# SYNTHESIS OF 3, 4, 5-TRIIODOPHENYLPYRROLIDONE CARBOXYLIC ACID, A POSSIBLE ISOMER OF THYROXIN.

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## INTRODUCTION.

The formula proposed by Kendall (1) for thyroxin, the crystalline active principle of the thyroid gland, is open to various criticisms on chemical grounds. In the first place, the stability of the compound, particularly towards boiling alkali, is scarcely consistent with the supposed existence in the molecule of a 2,4,6-trihydro-2,4,6-triiodoindole nucleus. The supposition that the compound is an indole derivative is apparently based on the fact that, under certain conditions, it gives the pine-splinter reaction; but here again the other properties of the substance, its relative stability towards oxidizing agents and, when pure, towards acids, are not such as would be expected from a consideration of the known properties of indole derivatives of a somewhat similar constitution to that proposed for thyroxin.

A consideration of the properties of the substance, as described by Kendall, led to the idea that a more straightforward explanation of them would be afforded if the substance were a phenyl derivative of glutamic acid. One of the main features of the chemistry of thyroxin is that it is capable of existing in more than one form, not only showing a keto-enol tautomerism, but also occurring in an "open-ring" form with the addition of the elements of water. This phenomenon is ascribed by Kendall to the opening of the pyrrole ring between the nitrogen and $\alpha$-carbon atoms. Now it is known from the work of Foreman (2) that glutamic acid is converted with moderate ease to pyrrolidone carboxylic acid, and a similar change was found to take place with even greater

readiness with β-hydroxyglutamic acid by Dakin (3), the reverse change being brought about by hydrolysis with strong hydrochloric acid. It seemed not improbable that the change triiodophenylglutamic acid⇄triiodophenylpyrrolidone carboxylic acid would take place with ease; and that such a constitution might, therefore, explain this characteristic of thyroxin.

Further, it is known that, under conditions which favour reduction, *e.g.* heating of their sodium salts, both glutamic and pyrrolidone carboxylic acids will give the pine-splinter reaction for pyrrole. The conditions under which Kendall obtains the pine-splinter reaction with thyroxin involve heating the sodium salt, so that here again the behaviour of thyroxin would be imitated by a phenylpyrrolidone carboxylic acid. Finally, the formula of a triiodophenylpyrrolidone carboxylic acid is $C_{11}H_8O_3NI_3$, whereas that of thyroxin, according to Kendall, is $C_{11}H_{10}O_3NI_3$. The analysis figures which Kendall publishes would, however, fit either of these formulas equally well, the difference in the percentage of the hydrogen in the two cases being within the limits of experimental error.

In view of these considerations it was thought to be worth while to prepare a triiodophenylpyrrolidone carboxylic acid with the object of determining whether such a substance would show chemical and physiological similarities to thyroxin.

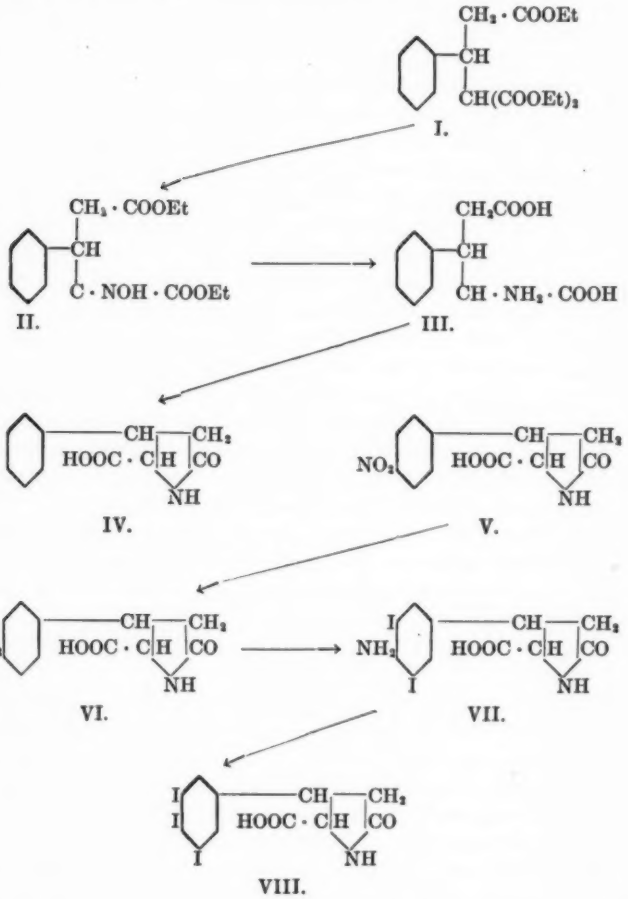
It may be stated at once that the substance prepared has not, so far as has been determined, any similar physiological action to that of thyroxin. It was not possible to observe any marked effects after the subcutaneous injection of large doses (50 to 100 mg.) into dogs. When administered to a normal individual on two occasions, (*a*) 25 mg. by the mouth, and (*b*) 12 mg. intravenously, it appeared to produce a slight increase in the basal metabolic rate. Another normal, however, showed no such effect on receiving a similar intravenous dose, and a cretin, who had been allowed to drop to −20 per cent basal metabolic rate, by ceasing to administer thyroid extract, showed no response to an intravenous dose of 12 mg. of triiodophenylpyrrolidone carboxylic acid, though on receiving 12 mg. of thyroxin (Squibb) her basal metabolic rate rose to + 12 per cent. This last test was considered crucial and it was concluded therefrom that, at least in doses of similar magnitude, the activity of the synthetic product

is nil as compared with that of thyroxin. In spite of this negative physiological result it is thought to be worth while describing briefly the synthesis, which offers some points of chemical interest.

In undertaking this synthesis the first substance aimed at was β-phenylglutamic acid. It was at first attempted to prepare this by the action of ammonia on α-bromo-β-phenylglutaric acid. The latter acid, which had not been prepared before, was obtained by the following series of reactions: ethyl cinnamate and ethyl malonate were condensed in the presence of sodium by Michael's reaction (4); the resulting tribasic ester was hydrolysed and the free malonic acid derivative brominated in ethereal solution; the bromomalonic acid derivative so obtained, on boiling in xylene, lost carbon dioxide and gave the desired α-bromo-β-phenylglutaric acid. Unfortunately, however, the replacement of Br by $NH_2$ in this compound does not proceed smoothly, owing to the ease with which HBr is split off. Aqueous and alcoholic ammonia, both at ordinary temperature and in the heat, were tried unsuccessfully as was also the phthalimide method. On allowing to stand at room temperature for 2 to 3 days with liquid ammonia, a small yield (5 to 10 per cent) of the amino acid was obtained. Since this synthesis was obviously almost impracticable, attention was directed to other modes of attack. The synthesis finally evolved takes the following course. The tribasic ester (I), obtained by the condensation of ethyl cinnamate with ethyl malonate, gives, with sodium ethoxide and ethyl nitrite, α-isonitroso-β-phenylglutaric ester (II); this can be hydrolysed to α-isonitroso-β-phenylglutaric acid and the latter, on reduction with sodium amalgam, gives β-phenylglutamic acid (III).

Having obtained this acid the next step was to investigate the ease with which it could be transformed into phenylpyrrolidone carboxylic acid. It was found that if the amino acid were boiled for a few hours in 10 per cent aqueous solution it gave a product which contained no amino nitrogen, and which proved in fact, on further analysis, to be phenylpyrrolidone carboxylic acid. It was obtained, moreover, in 80 to 90 per cent yield, and, on heating with hydrochloric acid, passed readily back to the amino acid. In view of the facts mentioned in the introduction, the great ease of this transformation was encouraging, and attempts

were at once made to prepare iodine derivatives. No direct method was found by which iodine could be introduced into the molecule, and resort had therefore to be had to nitration in order to render the benzene nucleus more susceptible to attack.




On nitration, phenylpyrrolidone carboxylic acid (IV) gives almost exclusively the *p*-nitro derivative (V). This can be

reduced by means of ferrous sulfate and baryta to the *p*-amino derivative (VI), which in turn, on treatment with an alkaline solution of iodine, takes up 2 atoms of iodine. By analogy with *p*-aminobenzoic acid these iodine atoms will have entered the 3,5 positions. On diazotising the resulting 3,5-diiodo-4-amino-phenylpyrrolidone carboxylic acid (VII) in strong sulfuric acid and treating the product with potassium iodide the amino group is displaced by iodine with the formation of 3,4,5-triiodophenyl-pyrrolidone carboxylic acid (VIII).

On obtaining this acid in a state of purity it was at once obvious from its physical properties that it was not identical with thyroxin. As had been anticipated, however, it gave the indole reaction under conditions described by Kendall. It was tested physiologically with the results mentioned above.

## EXPERIMENTAL.

*Condensation of Ethyl Cinnamate and Ethyl Malonate.*—This reaction was carried out as described by Vorländer (5), the esters being mixed in molecular proportions, treated with $\frac{1}{10}$ atom of metallic sodium, and heated for 2 hours in the boiling water bath. The free ester was liberated by shaking with dilute sulfuric acid, extracted with ether, dried, and distilled under reduced pressure. It distils at about 220° at 15 mm. of Hg and was obtained in a yield of 75 per cent of the theoretical.

*α-Isonitroso-β-Phenylglutaric Ester.*—15 gm. of the condensation product of ethyl cinnamate and ethyl malonate were mixed at 0° with 5 gm. of ethyl nitrite, and the mixture was added slowly, with careful mixing, to a solution of 1 gm. of sodium in 20 cc. of absolute alcohol. The flask and freezing mixture were left overnight in the ice chest, so that in the morning the temperature had not risen above −5°. The alcohol was then removed in a vacuum desiccator over sulfuric acid and the dark brown viscous residue dissolved in a little water; the solution was shaken with ether to remove some undissolved oil and the ester was then precipitated by passing carbon dioxide through the solution until saturated. After extraction with ether, drying, and removing the ether by distillation, the isonitroso ester was obtained as a brown oil. It did not crystallise, even on long standing in a vacuum desiccator,

nor could it be distilled. The best purification was effected by repeated solution in sodium hydroxide and reprecipitation with carbon dioxide. After several such treatments the colour became appreciably lighter. The yield of crude ester was 50 to 60 per cent of the theory.

*Analysis.* 0.1242 gm.: 0.2816 gm. $CO_2$ and 0.0774 gm. $H_2O$.
Found C 61.8, H 6.9.
Calculated for $C_{15}H_{19}O_5N$. " 61.4, " 6.5.

Attempts to hydrolyse and reduce this ester in one operation by the use of sodium in alcohol, resulted only in a small yield of β-phenylglutamic acid.

*α-Isonitroso-β-Phenylglutaric Acid.*—5 gm. of β-isonitroso-α-phenylglutaric ester were added to a solution of 10 gm. of KOH in 8 cc. of water. The mixture was warmed gently, with vigorous shaking, until the first reaction was over, and then further heated on the water bath for 5 minutes. It was cooled, diluted, acidified with hydrochloric acid, and thoroughly extracted with ether. The dried ethereal extract, on evaporation, yielded an oil which readily crystallised on rubbing with warm benzene. The yield was 4 gm. (practically theoretical). The acid was recrystallised by dissolving in glacial acetic acid and diluting the hot solution with benzene; on cooling, it separates in colourless blunt needles or elongated prisms, m.p. 129-130° with decomposition.

*Analysis.* 0.1222 gm.: 0.2492 gm. $CO_2$ and 0.0530 gm. $H_2O$.
0.2409 " : 12.2 cc. moist $N_2$ at 21° and 754 mm.
Found. C 55.61, H 4.81, N 5.70.
Calculated for $C_{11}H_{11}O_5N$. " 55.70, " 4.64, " 5.88.

*β-Phenylglutamic Acid.*—20 gm. of α-isonitroso-β-phenylglutaric acid were dissolved in 2,000 cc. of water and the solution was treated with 2,000 gm. of 2.5 per cent sodium amalgam in portions, the whole being stirred mechanically. At the end of about 3 hours the reduction was complete. The solution was then concentrated *in vacuo* to about 500 cc., the temperature being kept below 50°, well cooled, and cautiously made acid to Congo red with nitric acid; it was shaken with ether, which removes some coloured impurity; it was then made just alkaline to litmus with sodium hydroxide and treated with 20 per cent silver nitrate until no further precipitate was formed; the heavy white precipitate

was filtered off, thoroughly washed by grinding with water and refiltering twice, suspended in distilled water, and decomposed with hydrogen sulfide. The silver sulfide was filtered off and washed with hot water, the filtrate and washings being then concentrated to a small volume *in vacuo*. Traces of free mineral acid were removed by the very careful addition of sodium hydroxide until no longer acid to Congo red; on standing there crystallised out 13 gm. (70 per cent yield) of almost pure phenylglutamic acid. For complete purification it was crystallised once from water in which it is little soluble in the cold and from which it separates in colourless rhombic plates. The acid does not have a sharp melting point but becomes pasty at about 170° and melts with loss of water at 179°.

*Analysis.* 0.1613 gm.: 0.3498 gm. $CO_2$ and 0.0868 gm. $H_2O$.
Found. C 59.14, H 5.98, N 6.22, $NH_2N$ 6.18.
Calculated. " 59.19, " 5.83, " 6.28, " 6.28.
13.5 mg.: 0.8 mg. ammonia N (micro Kjeldahl).
13.5 " : 1.52 cc. $N_2$ at 21° and 756 mm. (Van Slyke).

The acid is slightly soluble in cold water, more readily in hot, and insoluble in alcohol. No specially characteristic metallic salt was found, so for further characterisation the acid was converted into the benzoyl derivative.

*α-Benzoylamino-β-Phenylglutaric Acid.*—On attempting to benzoylate β-phenylglutamic acid by Fischer's modification (6) of the Schotten-Baumann reaction a poor yield of the benzoyl derivative was obtained; better results were achieved as follows: 2.23 gm. of β-phenylglutamic acid were dissolved in a solution of 2.23 gm. of sodium acetate in 25 cc. of water which was made alkaline to litmus with sodium hydroxide; benzoyl chloride (3.5 cc.) and 2 N sodium hydroxide were then added alternately with vigorous shaking, the reaction being kept constantly just alkaline to litmus. At the end of the reaction the solution was made acid to Congo red with concentrated hydrochloric acid. After standing overnight the precipitate was filtered off, thoroughly dried, and repeatedly boiled out with ligroin to remove benzoic acid. The yield of crude α-benzoylamino-β-phenylglutaric acid amounts to 2.35 gm. (72 per cent of the theory). It was recrystallised from hot water, from which it is inclined to

separate as an oil, subsequently crystallising in colourless needles which melt at 171–172° after slight preliminary sintering.

*Analysis.* 19.6 mg.: 0.82 mg. ammonia N (micro Kjeldahl).
Found. N 4.19.
Calculated for $C_{18}H_{17}O_5N$. " 4.28.

The acid is very slightly soluble in cold water, more so in hot; it is easily soluble in alcohol and moderately so in ether. It titrates as a dibasic acid to phenolphthalein; 0.2318 gm. required 13.9 cc. of 0.1 N NaOH. Molecular weight found 334, calculated 327.

*Phenylpyrrolidone Carboxylic Acid.*—10 gm. of β-phenylglutamic acid in 100 cc. of water were boiled under a reflux condenser for 6 hours. On cooling there separated out about 8 gm. of a white crystalline substance which was soluble in alcohol and did not give the ninhydrin reaction. The compound was filtered off, recrystallised from alcohol from which it separates in long needles and narrow prisms, melting at 196.5–197.5° with slight sintering.

*Analysis.* 0.1220 gm.: 0.2878 gm. $CO_2$ and 0.0614 gm. $H_2O$.
12.58 mg.: 0.84 mg. ammonia N (micro Kjeldahl).
12.58 " : 0.01 cc. $NH_2N$ at 21° and 756 mm.
Found. C 64.34, H 5.59, N 6.70, $NH_2$ N 0.
Calculated for $C_{11}H_{11}O_3N$. " 64.39, " 5.37, " 6.83, " 0.

0.49 gm. was boiled with 10 cc. of 50 per cent hydrochloric acid for 4 hours; the solution was cooled, neutralised, and made up to 50 cc. 2 cc. gave 1.26 mg. of ammonia N (micro Kjeldahl) and 2.16 cc. of $N_2$ at 18° and 762 mm. (Van Slyke) = 1.24 mg. of amino N. The extent of reconversion to β-phenylglutamic acid was therefore 98 to 99 per cent. The acid titrates as a monobasic acid to phenolphthalein; 0.241 gm. required 11.5 cc. of 0.1 N NaOH. Molecular weight found 209, calculated 205.

*p-Nitrophenylpyrrolidone Carboxylic Acid.*—2.9 gm. of phenylpyrrolidone carboxylic acid were added slowly to 15 gm. of fuming nitric acid, the temperature being kept at 0° or just below. The solution was then poured on to ice and the precipitate filtered off and well washed with water. It was dissolved in boiling 95 per cent alcohol from which on cooling and long standing there separated about 2 gm. of the *p*-nitro acid in colourless rectangular

XUM

plates. On recrystallisation from alcohol the acid darkened at 229° and melted at 252° with decomposition.

*Analysis.* 17.8 mg.: 1.98 mg. ammonia N (modified micro Kjeldahl) (see Stewart (7)).

Found. N 11.12.
Calculated for $C_{11}H_{10}O_5N_2$. " 11.20.

*p-Aminophenylpyrrolidone Carboxylic Acid.*—The reduction of the *p*-nitrophenylpyrrolidone carboxylic acid presented unexpected difficulties, neither tin and hydrochloric acid nor stannous chloride in alcohol nor ammonium sulfide giving satisfactory results. The following method was finally tried with success. A hot solution of 54 gm. of ferrous sulfate in 135 cc. of water was treated with a hot concentrated solution of 62 gm. of barium hydroxide; to the mixture was added a solution of 7.5 gm. of *p*-nitrophenylpyrrolidone carboxylic acid in a slight excess of 5 per cent barium hydroxide; the whole was well shaken and heated on the water bath for 10 minutes, cooled, and filtered, the precipitate being washed with hot water; the barium was exactly removed from the filtrate with sulfuric acid and the filtrate from the barium sulfate concentrated to small bulk *in vacuo*. The *p*-amino acid crystallised out almost pure in a yield of 4.5 gm. (68 per cent of the theory). On recrystallisation from 50 per cent alcohol it forms colourless needles which tend to be arranged in sheaves and melt at 254° with decomposition.

*Analysis.* 10.8 mg.: 1.35 mg. ammonia N (micro Kjeldahl).

Found. N 12.54.
Calculated for $C_{11}H_{12}O_3N_2$. " 12.72.

*3,5-Diido-4-Aminophenylpyrrolidone Carboxylic Acid.*—16 gm. of iodine were dissolved in just enough sodium hydroxide to give an almost colourless solution; to this was added a solution of 4.5 gm. of sodium iodate which had been treated with 1 equivalent of sulfuric acid and the whole was made up to a liter with water. 4.4 gm. of *p*-aminophenylpyrrolidone carboxylic acid were dissolved in 50 cc. of concentrated hydrochloric acid and 500 cc. of water; to this solution, warmed to 35° and vigorously agitated with a mechanical stirrer, was added the iodine solution from a burette. After the addition of about 1 molecular equiva-

lent of iodine a dark precipitate began to separate out, which increased in amount with the further addition of iodine up to 2 molecules. At this stage the addition of iodine was discontinued, the solution cooled, and the precipitate filtered off; it was dissolved in dilute ammonia and reprecipitated with $SO_2$, whereupon it separated almost colourless. It was crystallised from a large volume of 50 per cent alcohol, from which it separated in long glistening needles, which retained a faint straw colour and, on heating, darkened at 228° and melted at 234° with decomposition. The yield was 6.4 gm. (68 per cent of the theory).

*Analysis.* 0.2192 gm.: 0.2247 gm. $CO_2$ and 0.0456 gm. $H_2O$.
0.1347 " : 0.1345 " Ag I.
8.58 mg.: 0.492 mg. ammonia N (micro Kjeldahl).
Found. C 27.96, H 2.31, N 5.73, I 54.02.
Calculated for $C_{11}H_{10}O_3N_2I_2$. " 27.97, " 2.12, " 5.93, " 53.82.

*3,4,5-Triiodophenylpyrrolidone Carboxylic Acid.*—4.5 gm. of 3,5-diiodo-4-aminophenylpyrrolidone carboxylic acid were dissolved in 20 cc. of cold concentrated sulfuric acid and 1.75 gm. (2.5 mols) of finely powdered sodium nitrite were stirred in. The mixture was kept at 0° for 2 hours and then poured on to ice; the cold solution was treated with 9 gm. of potassium iodide dissolved in 20 cc. of water, which produced a red precipitate. The mixture was warmed on the water bath until the evolution of nitrogen ceased, cooled, treated with a little sodium bisulfite to remove free iodine, and filtered. The dark brown precipitate was dissolved in dilute ammonia, treated with just enough aqueous $SO_2$ to decolourise the solution, and the precipitation was completed by the addition of acetic acid. The precipitate was filtered off and crystallised from 80 per cent alcohol, from which it separated in colourless needles melting at 247° with decomposition.

*Analysis.* 0.2218 gm.: 0.1860 gm. $CO_2$ and 0.0340 gm. $H_2O$.
20.6 mg.: 0.48 mg. ammonia N (micro Kjeldahl).
0.1496 gm.: 0.1798 gm. Ag I.
Found. C 22.90, H 1.7, N 2.33, I 65.01.
Calculated for $C_{11}H_8O_3NI_3$. " 22.6, " 1.4, " 2.40, " 65.26.

The acid is moderately soluble in hot alcohol and practically insoluble in water. It gives the pine-splinter reaction faintly when fused with sodium hydroxide and with intensity on heating

with zinc dust. Owing to its great insolubility it is difficult to hydrolyse the acid to the corresponding glutamic acid derivative. However, on boiling for a short time with a large volume of 25 per cent hydrochloric acid evidence was obtained by the appearance of the ninhydrin reaction of the formation of an amino acid.

SUMMARY.

The synthesis is described of 3,4,5-triiodophenylpyrrolidone carboxylic acid, a substance having practically the same empirical formula as that of thyroxin as determined by Kendall, and which shows certain chemical similarities to the latter substance.

Physiological tests show the synthetic compound to be without effect on the basal metabolic rate.

For help in the physiological testing I am indebted to Dr. K. S. Hetzel.

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XUM

XUM

# THE BIOCHEMISTRY OF ACETONE FORMATION FROM SUGARS BY BACILLUS ACETOETHYLICUM.

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Acetone is found among the products of two distinct types of bacteriological fermentation. Anaerobic bacilli of the amylobacter type produce acetone and butyl alcohol from a large series of sugars, and the characteristic acid products are butyric and acetic acids. A second group of bacilli produce acetone and ethyl alcohol from sugars, and the volatile acids produced are acetic acid and formic acid. The first member of this group to be described was *Bacillus macerans* by Schardinger (1), and the second was isolated, described, and named by Northrop, Ashe, and Senior (2) *Bacillus acetoethylicum*. The two species differ in only one important characteristic; namely, *Bacillus acetoethylicum* alone is able to ferment galactose and levulose in a medium containing ammonium salts as the source of nitrogen. The investigation of this type of fermentation was continued by Arzberger, Peterson, and Fred (3, 4), and mention will be made later of several important points established by these workers. Neuberg and his associates have discussed the method by which acetone is produced by bacterial action, and they are of the opinion that the following scheme holds for both the amylobacter and macerans types of fermentation (5).

Acetaldehyde→aldol→acetoacetic acid→acetone

Northrop in his original paper reported that the neutral products of the fermentation of several sugars were 5 to 10 per cent acetone and 12 to 25 per cent ethyl alcohol, whereas, there was no acetone produced from glycerol, but the yield of ethyl alcohol rose to 40 per cent. An attempt has been made to study further these fermentations, to account for the difference in the products,



XUM

and to utilize the experimental evidence obtained in formulating a biochemical scheme for this organism.

## EXPERIMENTAL.

The parent culture used in this investigation was obtained by the courtesy of the American Museum of Natural History, New York. Stock cultures have been maintained for several years by periodic transfers in a medium containing maize meal and 1 per cent $CaCO_3$. For the purpose of these experiments active cultures were obtained in the following manner. Agar plate cultures grown aerobically were prepared from an active maize culture. Individual colonies were transferred to two types of liquid media, one containing 3 per cent maltose and the other 3 per cent glycerol in a stock solution of mineral salts, calcium carbonate, and peptone. For several weeks the organism was kept active in these two media in order to rule out from our glycerol experiments any changes due to an initial transfer of the bacillus from a medium containing sugar. Both types of media support active fermentations.

*Experiment 1.*—A preliminary qualitative examination was made of the products obtained by the fermentation of glucose, maltose, and glycerol. Two points of theoretical significance were established. Previous reports contain very little information regarding the nature of the gas which is evolved. Northrop does not mention the subject, and in the experiments of Arzberger, Peterson, and Fred the gas was estimated quantitatively as $CO_2$. That $H_2$ is evolved in sugar and glycerol fermentations can be shown by collecting samples of gas, removing the $CO_2$ by means of NaOH, and exploding the residue with air. This is in accordance with the facts relating to similar bacteriological fermentations.

The second point of importance is that traces of pyruvic acid are present at various times in fermenting media containing glycerol or glucose. This was first indicated satisfactorily during an examination of old test-tube cultures, using the method recommended by Simon and Piaux (6) and Quastel (7). To a small volume of culture add 2 cc. of $H_2O$ and supersaturate with solid $(NH_4)_2SO_4$ in the cold. Add a crystal of sodium nitroprusside and 2 cc. of $NH_4OH$. Shake and allow to stand at room temperature. A blue colour gradually develops, and in dilute solutions of pyruvic acid its intensity is proportional to the concentration of the acid. The test is essentially Rothera's test for acetone, and when this substance is present in the medium it is impossible to establish the presence of traces of pyruvic acid in the mixture owing to the purple colour which develops. This difficulty can be overcome in the following manner. A sample of the culture

XUM

to be tested is heated for 5 minutes at 50°C. and 20 mm. of pressure. A little capryl alcohol is added to prevent foaming. Acetone distills over without decomposition of any pyruvic acid which may be present. The test outlined above can be then applied to the residue. The blue colour obtained varies from a greenish blue to a pure indigo, according to the amount of pyruvic acid in solution, and for this reason difficulty was encountered when attempts were made to estimate pyruvic acid by the use of a standard solution and the colorimeter. A method has been devised, however, which has given satisfactory results in experiments during which the utilization of pyruvic acid by the bacillus was followed. The following standardized conditions are necessary. To 4 cc. of distilled $H_2O$ add 1 cc. of the culture and an excess of $(NH_4)_2SO_4$ crystals. Run in from a pipette 2 cc. of a 1 per cent solution of sodium nitroprusside, freshly prepared, and 1 cc. of $NH_4OH$. Allow to stand for 10 minutes, and shake at intervals. Compare the colour which develops with a series of test-tubes containing from 1 to 20 per cent of $CuSO_4$ in $H_2O$ by means of a block comparator. These tubes must be previously standardized in terms of pyruvic acid. In this way concentrations of pyruvic acid from zero to 0.2 per cent can be measured.

The remaining products found in our preliminary experiments were those which have been identified by previous workers.

*Experiment 2.*—In order to study more completely the occurrence of pyruvic acid in the fermentation, and its relation to acetone formation, the following experiment was performed. Three series of test-tubes containing maltose, glucose, and glycerol respectively in 3 per cent concentration were prepared, and inoculated. The basis of these media was a mineral salt solution containing also 0.5 per cent of peptone and 1 per cent of $CaCO_3$. The glycerol tubes were inoculated from stock glycerol cultures in an active condition, and the sugar-containing tubes from maltose cultures. They were incubated at 38°C., and at intervals a tube of each type was examined qualitatively for acetone and pyruvic acid. Observations regarding gas evolution were also made. The results are summarized in Table I.

In the tubes containing sugar pyruvic acid was present during the first half of the fermentation period. Later the free acid disappeared, and acetone accumulated. The maltose tubes were more active than the glucose ones, but the amount of pyruvic acid which could be detected was by far the greater in the glucose medium. In the glycerol fermentation a trace of pyruvic acid was present on the 5th day and more definite amounts on the 21st day of incubation. The loss of acetone in all the tubes was due to evaporation. There appeared to be two possible explanations for the almost complete absence of pyruvic acid and acetone in the products of the glycerol fermentation: (*a*) the organism only

produces the acid from glycerol with difficulty, and pyruvic acid being the intermediate in acetone formation, little of the latter takes place; and (*b*) pyruvic acid is formed readily from glycerol, but it is converted with equal rapidity into some other characteristic product, most probably ethyl alcohol.

TABLE I.

| Time after inoculation. | Maltose. | | | Glucose. | | | Glycerol. | | |
|---|---|---|---|---|---|---|---|---|---|
| | Acetone. | Pyruvic acid. | Gas. | Acetone. | Pyruvic acid. | Gas. | Acetone. | Pyruvic acid. | Gas. |
| *days* | | | | | | | | | |
| 1 | | | + | | | + | | | + |
| 2 | − | + | ++ | − | ++ | + | − | − | +++ |
| 3 | − | + | ++ | − | ++ | + | − | − | +++ |
| 5 | − | ++ | + | − | ++ | + | − | + | +++ |
| 7 | + | + | ++ | − | ++ | + | + | − | +++ |
| 9 | +++ | − | +++ | + | + | + | − | − | + |
| 12 | +++ | − | + | + | − | + | − | − | − |
| 21 | + | + | − | − | + | − | − | + | − |

TABLE II.

| Time after inoculation. | Maltose medium. | | Glycerol medium. | |
|---|---|---|---|---|
| | Acetone in 100 cc. | Ethyl alcohol in 100 cc. | Acetone in 100 cc. | Ethyl alcohol in 100 cc. |
| *days* | *gm.* | *gm.* | *gm.* | *gm.* |
| 2 | 0.0145 | 0.0828 | 0 | 0.0415 |
| 4 | 0.0459 | 0.1521 | 0 | 0.1449 |
| 6 | 0.0918 | 0.2277 | 0 | 0.2484 |
| 8 | 0.1596 | 0.3519 | Trace. | 0.3933 |
| 11 | 0.2263 | 0.4968 | 0.0158 | 0.6727 |
| 13 | 0.2635 | 0.5796 | 0.0333 | 0.9315 |
| 15 | 0.2901 | 0.6831 | 0.0333 | 1.1178 |
| 18 | 0.3108 | 0.7245 | 0.0314 | 1.1178 |
| Per cent of yield..... | 10.3 | 24.1 | 1.0 | 37.3 |

*Experiment 3.*—During our preliminary experiments in which *B. acetoethylicum* was grown for about twenty generations in a glycerol medium it was always possible to detect acetone in the products by qualitative methods. To obtain more definite information regarding the time and extent of this formation the following experiment was performed. Two flasks containing 1,500 cc. of 3 per cent maltose and 3 per cent glycerol

medium respectively were prepared, and sterilized in the usual way. Each contained an equal amount of filter paper, cut into thin strips. The flasks were inoculated with 200 cc. of an active culture in the same type of medium. Samples of 100 cc. were withdrawn at intervals under sterile conditions, and distilled. Quantitative measurements of acetone and ethyl alcohol were made, acetone by the Messinger method and ethyl alcohol by oxidation with potassium dichromate and sulfuric acid, followed by a titration of the excess dichromate with potassium thiosulfate. It is unnecessary to describe the methods of procedure in detail. These observations were continued until both fermentations had finished, and the results are given in Table II. This experiment was performed three times.

This experiment indicates that during the glycerol fermentation there is a small amount of acetone formed during the second half of the fermentation period. Samples of culture after the 8th day gave a definite purple colour when Rothera's test for acetone was made, showing that the quantitative measurements obtained by the iodoform method were not due to the ethyl alcohol. At no time during this experiment was it possible to detect pyruvic acid in either flask. These fermentations were more active than test-tube cultures, and these observations suggest that the occurrence of free pyruvic acid is determined not only by the nature of the substrate but also by the activity of individual fermentations.

*Experiment 4.*—Acting on the hypothesis that in the last experiment no acetone was formed in the early stages of the glycerol fermentation owing to the absence of pyruvic acid in the products, several experiments of the following general type were performed. Two flasks containing 1,500 cc. of glycerol medium were prepared and sterilized. To one flask was added just previous to its inoculation 100 cc. of $H_2O$ containing 4 gm. of pyruvic acid (Eastman Kodak Company), neutralized with NaOH. The solution of pyruvate had been sterilized by passage through a Berkefeld filter. Both flasks were inoculated with 200 cc. of an active culture in glycerol medium. At intervals during the period of incubation quantitative measurements of pyruvic acid, acetone, and ethyl alcohol were made. On the 2nd day we observed that almost the whole of the pyruvic acid had been utilized by the organism, and that already acetone had been produced. By the 4th day all the pyruvic acid had been destroyed, and the acetone content of the medium showed an increase of 200 per cent. Ethyl alcohol formation and gas production were also more rapid in the flask which contained pyruvic acid. On the 8th day of incubation a second quantity of pyruvic acid, 3 gm., was neutralized and sterilized. The solution contained 3 gm. of glycerol, and was made up to 100 cc. This was added to the flask which had received the first batch. The effect on the general appearance of the fermentation was very pronounced. Gas produc-

tion was greatly stimulated, and the surface of the medium was covered with filter paper and calcium carbonate. The pyruvic acid was again rapidly utilized, and the effects on the rates of formation of ethyl alcohol and acetone are indicated in Table III and Fig. 1.

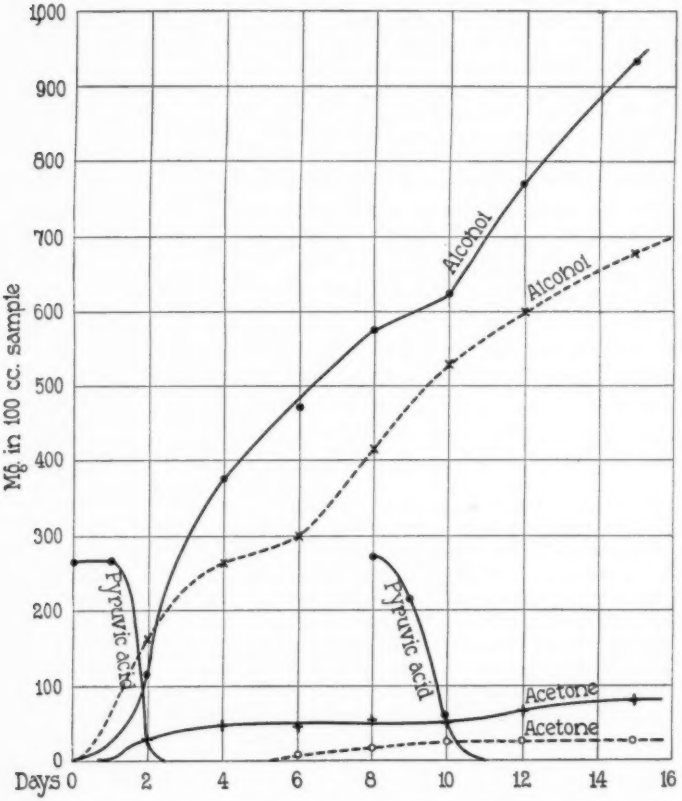


FIG. 1. Curves relating to two glycerol fermentations. To one flask, continuous curves, pyruvic acid was added at the commencement of and during the fermentation.

The information obtained by these experiments justifies several important conclusions. In the first place it is clear that *Bacillus acetoethylicum* utilizes very rapidly any free pyruvic acid in a

glycerol medium. This results in the formation of acetone earlier and larger in amount than in the control flask. The two processes do not coincide in time, but about half of the acetone formation takes place after pyruvic acid has disappeared from the medium. These facts suggest that in the formation of acetone from pyruvic acid there are intermediates formed which accumulate. The

TABLE III.

| Time after inoculation. | Glycerol. | | Glycerol + pyruvic acid. | | |
|---|---|---|---|---|---|
| | Acetone in 100 cc. | Ethyl alcohol in 100 cc. | Acetone in 100 cc. | Ethyl alcohol in 100 cc. | Pyruvic acid in 100 cc. |
| *days* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 0 | — | — | — | — | 0.266 |
| 1 | — | — | — | — | 0.266 |
| 2 | 0 | 0.1610 | 0.0263 | 0.1150 | 0.025 |
| 4 | 0 | 0.2645 | 0.0465 | 0.3795 | 0 |
| 6 | 0.0077 | 0.2990 | 0.0465 | 0.4715 | 0 |
| 8 | 0.0180 | 0.4140 | 0.0516 | 0.5750 | 0 |
| 8 | | | 0.0516 | 0.5750 | 0.273 |
| 9 | — | — | — | — | 0.218 |
| 10 | 0.0258 | 0.5290 | 0.0516 | 0.6210 | 0.060 |
| 12 | 0.0258 | 0.5980 | 0.0671 | 0.7705 | 0 |
| 15 | 0.0258 | 0.6747 | 0.0800 | 0.9352 | 0 |

TABLE IV.

| Nature of substrate. | Amount of final products present in 100 cc. | | |
|---|---|---|---|
| | Acetone. | Ethyl alcohol. | Volatile acid. |
| | *gm.* | *gm.* | *gm.* |
| Glycerol | 0.0116 | 0.598 | 0.050 |
| Pyruvic acid | 0.0725 | 0.207 | 0.410 |
| Mixture | 0.0580 | 0.506 | 0.230 |

actual yield of acetone from the pyruvic acid is not equal to that required by the scheme:

$$2CH_3\cdot CO\cdot COOH \rightarrow 2CH_3\cdot CHO \rightarrow CH_3\cdot CHOH\cdot CH_2\cdot CHO \rightarrow CH_3\cdot CO\cdot CH_3$$

From the amount of pyruvic acid added to the flask in this experiment we should expect to obtain 0.180 gm. of acetone per 100 cc., which with the amount formed in the control flask equals 0.205 gm. Roughly one-half of this amount was obtained, and it is

clear that from the pyruvic acid some other derivative is obtained, most probably ethyl alcohol. This conclusion is supported by the form of the ethyl alcohol curve in Fig. 1.

*Experiment 5.*—The purpose of this experiment was to determine the products of the fermentation of pyruvic acid, glycerol, and a mixture of the two. The following flasks were prepared, and sterilized.

Flask A. 200 cc. of mineral salt solution + 2 gm. glycerol + 1 gm. peptone + 2 gm. $CaCO_3$.

Flask B. 200 cc. of mineral salt solution + 2 gm. pyruvic acid + 1 gm. peptone + 2 gm. $CaCO_3$.

Flask C. 200 cc. of mineral salt solution + 1 gm. glycerol + 1 gm. acid + 1 gm. peptone + 2 gm. $CaCO_3$.

The pyruvic acid was in each case neutralized with NaOH, and sterilized by the filtration method. Each flask was inoculated with 10 cc. of an active culture in the glycerol medium. When fermentation had ceased in all the flasks certain quantitative and qualitative determinations were made after distilling 100 cc. portions. The results are given in Table IV.

The whole of the pyruvic acid in the mixture was utilized, but not in the pure pyruvic acid fermentation. The weight of volatile acid in each case was determined on the basis of equal weights of acetic and formic acids, a ratio which approximates to that found by Arzberger, Peterson, and Fred (3) for the products from glucose. Qualitative tests left no doubt that acetone is formed in large amounts from pure pyruvic acid.

## DISCUSSION.

The following is an attempt to summarize those observations of previous workers and those contained in this report which throw light on the biochemical nature of the fermentation of different media by *Bacillus acetoethylicum*.

1. Northrop, Ashe, and Senior point out that during the fermentation of glucose the ratio between the weights of acetone and ethyl alcohol in the products fell from 2.9 on the 3rd day to 2.2 on the 5th day, and continued at this figure until the end of the fermentation (2). This observation can be verified and amplified by considering the curve in Fig. 2 which is based on the results contained in Table II relating to the maltose fermentation. The form of the curve is an indication that at the commencement of the fermentation, and during several hours subsequently, the sole

neutral product is ethyl alcohol. Acetone formation commences later, and owing to its gradually increasing rate the ratio between the products falls to 2.2. These facts alone would suggest that acetone and ethyl alcohol are formed by independent biochemical processes, but we have shown in Experments 3 and 4 that both products are formed from pyruvic acid. Also the recurrence of the ratio 2.2 and its persistence for several days is significant.

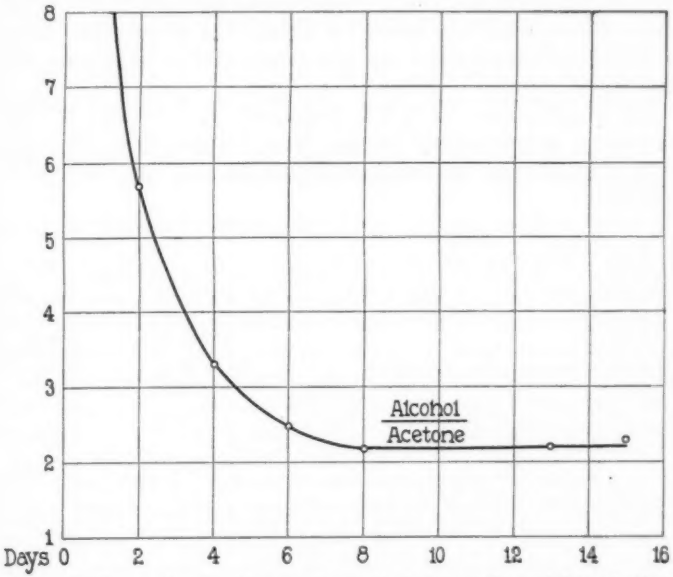


FIG. 2. Curve showing the varying ratio between the weights of ethyl alcohol and acetone in the products during the course of a maltose fermentation (Experiment 2).

The more justifiable conclusion would seem to be that ethyl alcohol has a 2-fold origin: (*A*) by a process in which acetone is not involved, and (*B*) a series of chemical changes by which definite quantities of both substances are produced. The curve in Fig. 2 according to this interpretation represents a gradual change from (*A*) to (*A* + *B*), at which point the ratio 2.2 is established.

2. The yields of acetone and ethyl alcohol from glucose are reduced, and the yield of volatile acid is increased when the acid

products are neutralized at regular intervals with NaOH.[1] This being the case it is not possible that ethyl alcohol is formed with acetic acid from acetaldehyde by the Cannizzaro reaction:

$$2\ CH_3 \cdot CHO + H_2O = CH_3 \cdot COOH + CH_3 \cdot CH_2OH$$

3. The composition of the volatile acid fraction obtained by the fermentation of sucrose and starch has been determined by Arzberger, Peterson, and Fred.[2] The figures in Table V are taken from their report, and below we include the percentage figures relating to equimolecular portions of the two acids, formic and acetic.

Both acids were produced from pure pyruvic acid by this organism, and probably during those fermentations in which pyruvic acid is an intermediate they are formed according to the equation:

$$CH_3 \cdot CO \cdot COOH + H_2O = CH_3 \cdot COOH + H \cdot COOH$$

TABLE V.

| | Formic acid. | Acetic acid. |
|---|---|---|
| | *per cent* | *per cent* |
| Sucrose | 40.55 | 59.55 |
| Starch | 43.32 | 56.68 |
| $H \cdot COOH + CH_3 \cdot COOH$ | 43.40 | 56.50 |

4. Lactic acid accumulates rapidly during the first stages of a glucose fermentation. The rate of production then falls to a constant at which it continues until the end of the fermentation. There is no evidence that lactic acid is an intermediate in the formation of other products.[3]

On the basis of the above experimental evidence the following scheme for the fermentation of glucose has been formulated.

$$\begin{array}{ccccc} & & CH_3 \cdot CHOH \cdot COOH & & \\ & & \uparrow + H_2 & & \\ C_6H_{12}O_6 & \longrightarrow & CH_3 \cdot CO \cdot COOH + H_2 & \longrightarrow & CH_3 \cdot CHO + CO_2 \\ & & \downarrow + H_2O & & \downarrow + H_2 \\ & & CH_3 \cdot COOH + H \cdot COOH & & CH_3 \cdot CH_2OH \end{array}$$

[1] Arzberger, Peterson, and Fred (3), p. 473.
[2] Arzberger, Peterson, and Fred (3), p. 470.
[3] Arzberger, Peterson, and Fred (3), p. 476.

This scheme is that designated (*A*) in Section 1 of this discussion. When acetaldehyde is produced more rapidly than it is being reduced, and therefore present within the cell in the free state, the following series of changes takes place.

$$2\ CH_3 \cdot CHO = CH_3 \cdot CHOH \cdot CH_2 \cdot CHO$$

Aldol.

$$CH_3 \cdot CHOH \cdot CH_2 \cdot CHO + \begin{matrix} O \\ | \\ H_2 \end{matrix} = CH_3 \cdot CHOH \cdot CH_2 \cdot COOH + CH_3 \cdot CH_2OH$$

$$CH_3 \cdot CHOH \cdot CH_2 \cdot COOH + \begin{matrix} O \\ | \\ H_2 \end{matrix} = CH_3 \cdot CO \cdot CH_2 \cdot COOH + CH_3 \cdot CH_2OH$$

$$CH_3 \cdot CO \cdot CH_2 \cdot COOH = CH_3 \cdot CO \cdot CH_3 \quad CO_2$$

Acetoacetic acid.

The question at once arises as to whether the above scheme for the glucose fermentation enables one to give an adequate explanation for the almost complete absence of acetone in the products of a glycerol fermentation, or in other words why does Section *B* of the scheme not come into operation? Let us consider the difference between molecules of glucose and glycerol, and assume that equal weights of pyruvic acid and acetaldehyde are produced from them.

$$C_6H_{12}O_6 = 2\ C_3H_4O_3 + 2H_2 = 2\ CH_3 \cdot CHO + 2CO_2 + 2H_2$$
$$2\ C_3H_8O_3 = 2\ C_3H_4O_3 + 4H_2 = 2\ CH_3 \cdot CHO + 2CO_2 + 4H_2$$

By the change from a glucose to a glycerol substrate you change the mass relationship between acetaldehyde and $H_2$, and the process of reduction to ethyl alcohol is thereby facilitated. Acetaldehyde rarely occurs free within the cell during the glycerol fermentation, and consequently aldol condensation and acetone formation do not take place. When pyruvic acid is added to the glycerol medium it is fermented more rapidly than the glycerol, and a new relationship $CH_3 \cdot CHO : H_2$ is established which approximates more to that obtaining during the latter half of a glucose fermentation, and consequently acetone formation occurs.

We have shown that the bacillus is able to form acetone from pure pyruvic acid. If this process involves aldol condensation

followed by oxidation it is necessary to postulate some acceptor for $H_2$. The evidence points to acetaldehyde as this substance.

The general significance of this type of work in the larger field of carbohydrate oxidation has been adequately treated in recent reviews by Dakin (8) and Shaffer (9).

SUMMARY.

1. The fermentation of glucose, maltose, and glycerol by *Bacillus acetoethylicum* has been studied from the point of view of acetone and ethyl alcohol production.

2. Pyruvic acid has been shown to be an intermediate compound in these fermentations.

3. Pure pyruvic acid is fermented, and from it acetone, ethyl alcohol, and volatile acids are formed.

4. A biochemical scheme for these fermentations has been postulated.

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XUM

# A METHOD OF OBTAINING FROM VEINS BLOOD SIMILAR TO ARTERIAL BLOOD IN GASEOUS CONTENT.*

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(Received for publication, March 6, 1925.)

The marked effect of external and internal influences upon the blood flow through the peripheral parts of the body, and the consequent variability of the gaseous content of the venous blood from these areas, have led to the use of arterial blood for studies involving the blood gases. Aside from the necessity of ruling out the factors just mentioned, it frequently becomes necessary to ascertain the oxygen saturation and carbon dioxide content of arterial blood.

The procedure of arterial puncture in adult man may be carried out in most cases without difficulty, and is apparently free from danger. However, the insertion of a needle into an artery may be more painful, and is not as easy or as certain of accomplishment as puncture of a superficial vein. Also, the number of times which blood may be safely drawn from an artery within short intervals is limited.

A procedure which would eliminate the necessity for arterial puncture and still give a blood with an arterial gaseous content would be useful. The method to be described has as its basis the two observations which follow.

It has long been known that if the arm is exposed to a sufficiently high temperature the skin becomes hyperemic; there is a marked vascular dilatation, hence an increased volume of blood in the vessels (1), as well as a more rapid flow through the part (2).

* The substance of this paper was presented before the American Society of Biological Chemists at Washington on December 30, 1924 (Goldschmidt, S., and Light, A. B., *J. Biol. Chem.*, 1925, lxiii, p. xxxviii).

Although this procedure markedly increases the oxygen content of blood from the veins at the bend of the elbow (3, 4), individuals have been encountered in whom it was possible to reach only the lower range of the oxygen saturation of arterial blood. The probable reasons are discussed elsewhere (4).

We have found (5) that, under ordinary bodily conditions, the blood from the veins on the dorsal surface of the hand has a higher average oxygen saturation and a lower carbon dioxide content than has blood from the veins in the antecubital space. In fact, in many subjects, the former blood approximates arterial blood in its gaseous content. However, the average oxygen saturation (88 per cent) of a large number of determinations is below the arterial range. Lundsgaard and Möller (6) concluded that "mixed cutaneous blood," obtainable in small amounts from one or more incisions on the finger-tip, may show almost identical values of oxygen content as arterial blood. They discuss the possibility of the substitution of the one for the other.

Neither of the above findings alone satisfied the requirements of a method adapted to yield from veins blood of an arterial gaseous content. By combining the two, it has been found possible to obtain from the veins on the dorsal surface of the hand, after immersion in hot water, blood indistinguishable from arterial blood in its oxygen content, capacity, and carbon dioxide content. Whether this blood is similar in respect to its content of other constituents, which are said to vary between arterial and venous blood, has not been determined. Of the two factors which contribute to make the hand especially suitable for obtaining these results, the first is the high oxygen saturation and low carbon dioxide content of the blood normally coursing through the veins of this part. This, we believe, is indicative of the presence of conditions insuring a rapid blood flow through tissues low in metabolism. The second is the relative superficiality of the vessels, allowing the heat to be effective upon the greater proportion, if not all the vessels. These points are elaborated in another paper (4).

*Technique of Method.*

The procedure adopted in obtaining the results reported herein is as follows. The hand and wrist, to a depth of about 3 inches

above the radial styloid, are immersed in water at a temperature of 45–47°C. The water is stirred frequently, and the temperature is maintained within this range by the addition of hot water. The temperature and time of exposure necessary to produce the desired results undoubtedly vary in different individuals. The interval of exposure and temperatures given have always resulted successfully under diverse conditions. At the end of 10 minutes, the hand is removed from the water a distance sufficient to allow insertion of the needle into a vein on the back of the hand. The fingers may be allowed to remain in the water and the blood is drawn immediately. A site of puncture close to the knuckles is preferable. The needle is introduced with the point directed towards the fingers. The veins, distended by heat, are easily entered with the needle, and the outflow is rapid. A subject who lacked superficial veins in this area has never been encountered by us. A No. 16 gauge needle has proved satisfactory. Care must be taken not to pass through the opposite wall of the vein; hematomas rapidly form and obliterate the vein completely. Upon removal of the needle it is also advisable to apply a pressure pad. This precaution allows of repeated punctures in the same area within brief intervals.

## EXPERIMENTAL METHODS.

The blood in all the experiments here reported was drawn by gentle aspiration directly under paraffin oil into a tube containing potassium oxalate sufficient to make a final concentration of 0.3 per cent. An oxalate solution was introduced into the container and dried upon its walls by a current of air. The apparatus into which the blood was drawn was essentially that described by Van Slyke and Cullen (7).

The arterial blood was taken either from the radial or brachial artery by the introduction of a needle through the skin (8).

The determinations were in all cases performed at once in duplicate by independent workers, using the method of Van Slyke and Stadie (9). The fine bore constant pressure apparatus with a mechanical shaker was employed. 2 cc. of blood were used to determine the oxygen content and capacity respectively, and 1 cc. for carbon dioxide content.

XUM

EXPERIMENTAL.

In Table I are presented nine determinations of the oxygen content, capacity, and saturation of hemoglobin of blood drawn by the method described above. The oxygen saturations found are within the range of figures obtained for arterial blood by other observers, using the same method of analysis (10, 11, 12). Subject Murph. has an oxygen saturation on the lower limit of this

TABLE I.

*Effect of Local Heat upon the Oxygen Content and Saturation of Hemoglobin of Blood from Veins on the Dorsal Surface of the Hand.*

| Subject. | Date. | $O_2$ content of Hb. | $O_2$ capacity of Hb. | $O_2$ saturation of Hb. | $O_2$ unsaturation of Hb. | Temperature of water and time of immersion. |
|---|---|---|---|---|---|---|
| | *1923* | *vol. per cent* | *vol. per cent* | *per cent* | *vol. per cent* | |
| Hitch. | May 3 | 16.05 | 16.57 | 96.8 | 0.52 | 45–44°C. for 15 min. |
| | *1924* | | | | | |
| McK. | June 11 | 19.50 | 20.10 | 97.0 | 0.60 | 44–44.5°C. for 15 min. |
| Ad. | " 12 | 17.80 | 18.95 | 94.0 | 1.15 | 44.5–45.5°C. for 15 min. |
| Thoe. | " 17 | 18.67 | 19.18 | 96.3 | 0.51 | 45–46°C. for 15 min. |
| Baz. | " 18 | 18.37 | 18.74 | 98.0 | 0.37 | 45.5–46.5°C. for 15 min. |
| Lov. | " 19 | 16.96 | 17.87 | 94.9 | 0.91 | 43.5–46°C. for 10 min. |
| Tych. | " 21 | 19.73 | 20.09 | 98.2 | 0.36 | 45.5–46.5°C. for 9 min. |
| Murph.* | " 23 | 18.56 | 20.00 | 92.8 | 1.44 | 45.5–46.5°C. for 10 min. |
| | *1922* | | | | | |
| Dew. | June 29 | 19.26 | 21.15 | 91.0 | 1.89 | Normal venous blood. No heat. |
| | | 20.32 | 20.88 | 97.3 | 0.56 | Blood from same vein and site. 40–42°C. for 15 min. |

* Chronic cough.

range, yet comparable figures for arterial blood are to be found (12). As noted, this subject suffered from a chronic cough.

A comparison was made of the oxygen content, capacity of hemoglobin (6 subjects), and the carbon dioxide content (5 subjects) of arterial blood, with similar analyses of blood obtained from the veins of the hand after exposure to heat (Table II).

Four of the subjects were in good health, while the last two were hospital patients. All of the figures with the exception of those in the last experiment, both for oxygen and carbon dioxide,

are identical within the limits of error of the methods of analysis. The oxygen capacity was determined in each sample of blood. Subject Ruth (last in Table II) has a higher oxygen saturation and

TABLE II.

*Comparison of the Oxygen Content and Saturation of Hemoglobin, and the Carbon Dioxide Content of Blood from an Artery, and from a Vein at the Back of the Hand after Local Heat.*

| Subject. | Date. | $O_2$ content. | $O_2$ capacity. | $O_2$ saturation. | $O_2$ unsaturation. | $CO_2$ content. | Remarks. |
|---|---|---|---|---|---|---|---|
| | *1924* | *vol. per cent* | *vol. per cent* | *per cent* | *vol. per cent* | *vol. per cent* | |
| S. G. | June 13 | 20.08 | 20.62 | 97.4 | 0.54 | | Venous blood. Water at 45–46°C. for 15 min. |
| | | 20.33 | 20.75 | 98.0 | 0.42 | | Arterial blood. |
| A. B. L. | " 20 | 21.20 | 21.98 | 96.4 | 0.78 | 46.7 | Venous blood. Water at 45–46.5°C. for 11 min. |
| | | 21.13 | 21.91 | 96.4 | 0.78 | 47.5 | Arterial blood. |
| Ths. | " 24 | 19.48 | 20.13 | 96.8 | 0.65 | 49.2 | Venous blood. Water at 45.5–46.5°C. for 10 min. |
| | | 19.25 | 19.94 | 96.6 | 0.69 | 49.5 | Arterial blood. |
| Lov. | " 25 | 17.38 | 17.85 | 97.4 | 0.47 | 47.8 | Venous blood. Water at 45.5–46.5°C. for 11 min. |
| | | 17.08 | 17.41 | 98.1 | 0.33 | 47.9 | Arterial blood. |
| F. G.* | July 19 | 17.60 | 18.89 | 93.2 | 1.29 | 49.6 | Venous blood. Water at 45.5–46.5°C. for 10 min. |
| | | 17.60 | 18.83 | 93.5 | 1.23 | 49.9 | Arterial blood. |
| Ruth.† | " 21 | 16.03 | 32.04 | 50.0 | 16.01 | 29.7 | Venous blood. Water at 45–46.5°C. for 11 min. |
| | | 14.56 | 32.15 | 45.3 | 17.59 | 31.4 | Arterial blood. |

* Ulcerative colitis. Acrocyanosis, cause unknown.

† Pulmonary stenosis with patulous ductus Botalli.

a lower carbon dioxide content of the blood from the veins than in that from the artery. This is undoubtedly to be explained by the fact that, at the time of the arterial puncture, the subject

was exceedingly disturbed and held her breath. As a consequence, the cyanosis which was very marked became even more intense. The subject had a pulmonary stenosis with a patent ductus arteriosus. On the other hand, the insertion of the needle into the vein, previously performed, was taken very calmly. This case illustrates that full reliance cannot be placed upon arterial blood obtained under these conditions.

SUMMARY.

A method is described and results of its use are presented, by which blood of arterial oxygen content, capacity, and carbon dioxide content may be obtained from the veins on the back of the hand, after exposure to hot water.

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XUM

# ON PAPAIN LIPASE.

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It is well known that during germination of oil seeds an alteration of their lipase occurs. Contrary to older views, especially those of Connstein, Hoyer, and Wartenberg (1), the following explanation for this phenomenon is offered by Willstätter and Waldschmidt-Leitz (21, pp. 163, 207; 23, p. 145). In the resting seed the lipase exists as "spermatolipase" (seed lipase) and is absolutely inactive in a neutral medium (pH 6.8). Germination sets in with the hydrolysis of the seminal proteins. As soon as this has attained a certain degree, the seminal enzymes of the peptic group begin to act on the lipase. Through the action of these enzymes the spermatolipase is changed into another lipase, the "blastolipase" (germ lipase). This blastolipase possesses the ability, in contrast to the spermatolipase, to split fats also in a neutral medium. Only now can mobilization of the fat reserves be started by the blastolipase. It is noteworthy that this characteristic proteolysis of the spermatolipase to blastolipase during germination is produced by the enzymes of the peptic group, but not by tryptic ferments (23, p. 146; 21, p. 209).

But the effect of papain, as well as of papain activated by HCN, on spermatolipase has not been studied from this point of view. This investigation would be of particular interest, because Willstätter and Grassmann, in their noteworthy communication, were able to show that papain alone and papain activated by HCN behave like two specific enzymes from different proteolytic groups. Papain alone acts like pepsin, while "papain cyanhydrin" has a tryptic action; *i.e.*, peptone-forming as well as peptone-splitting (13, pp. 186, 205).

While investigating the action of papain and of papain cyanhydrin[1] on spermatolipase, it occurred to us that practically noth-

[1] We shall report on this question in the near future.

ing is known of the presence of a lipase in crude papain. It is true that Gerber (5, 6) has found lipases in the latex of several plants, but he did not investigate the latex of *Carica papaya*, the source of papain. Pratt (9, p. 31) refers to the possible existence of a lipase in papaya latex, but was not able to establish definitely its presence.

We found in crude papain a lipase of considerable activity. The properties of this papain lipase are briefly given.

Papain lipase splits both higher glycerides and lower esters. It is insoluble in water and has its optimum activity at 35–40°C. at a pH of 5.8 to 6.2 in acetate buffer. As to the kinetics, the law of Schütz is followed to a certain extent. HCN and bile salts have no effect at all on the activity of the lipase. Calcium chloride, however, accelerates it in an alkaline medium (changing pH).

Papain lipase differs from castor lipase in its pH optimum and its susceptibility to activation by calcium chloride (21, pp. 163, 167, 171). But is this difference in itself sufficient reason to assume two specific lipases? To answer this question we have to consider the recent development of views on the specificity of animal lipases. Loevenhart (8) was the first to find that the most essential difference between the lipases of the liver and the pancreas consists in their greatly differing "quantitative specificity" (15, p. 24; 17, p. 231) for substrates of different constitution. These results of Loevenhart were recently reinvestigated and their theoretical foundation (15) was broadened by Willstätter and his coworkers. Willstätter was able to improve the accuracy of Loevenhart's estimations by the comparative use of crude and purified lipase preparations and by eliminating the possibility of the influence of accompanying bodies by having the enzyme act under conditions that are made comparable by the addition of activators or of inhibiting substances (19, p. 231; 22, p. 210; 23, p. 146). But even a considerable difference in the quantitative specificity of two lipases, while suggestive, does not in itself suffice to prove finally their dissimilarity. Stereochemical specificity, however, seems really to constitute a property of the enzyme and to be independent of accompanying bodies (2; 3; 14, p. 206; 18; 19, p. 221). As such exact and comparable estimations have not yet been carried out for castor and papain lipase, the difference in their pH optimum and in their susceptibility to activation does not

*per se* suffice to decide whether or not they are really two different enzymes, for Willstätter, in his work with animal lipases, found that the dependence of lipolysis on hydrogen ion concentration, as it is experimentally found, is deceptive and is caused by other more complex phenomena (22, p. 97). Accompanying bodies influence the lipase in its relation to hydrogen ion concentration, to adsorption, activation, inhibition and poisoning, stability, optimum of temperature, and heat destruction (14, p. 205).

A lipase preparation obtained directly from papaya latex might be more suitable for investigating the relative specificity of castor and papain lipase.

## EXPERIMENTAL.

### *1. Estimation of the Lipolytic Action.*

In estimating the lipolysis, an olive oil of the saponification number 183 was usually employed as substrate.[2] The determinations were done in accord with Willstätter in the following manner (22, pp. 103, 111; 21, p. 177).

The papain preparation which is to be determined is weighed into a small wide mouthed bottle of a capacity of about 30 cc. with well ground stopper, then the desired amount of water or buffer solution is added,[3] and the mixture shaken for a short time, till the greater part of the papain has gone into solution. Now 2.5 gm. of olive oil are added, the mixture is shaken by hand strongly and evenly for 3 minutes (22, pp. 104, 112; 21, p. 166) and left in the thermostat at the desired temperature for the remaining time of the estimation. After the reaction has gone on for a given time, it is stopped by bringing the saponification mixture into an Erlenmeyer flask with the help of about ten times the amount of 95 per cent alcohol (distilled over KOH) and 20 cc. of ether. Then the fatty acid which has been formed is estimated, using 15 drops of a 1 per cent alcoholic solution of thymolphthalein as indicator (22, p. 105), by titration with 0.5 N alcoholic KOH (alcohol content 90 per cent), till a distinctly blue color appears.

[2] Methyl butyrate according to Willstätter (17, p. 236): volume 20 cc.; 5 cc. of N acetate buffer (pH 6.8), 0.2 gm. of methyl butyrate, 100 mg. of papain (Wallerstein), 60 min., 30°C. = 9.1 per cent cleavage.

[3] The lipase being insoluble in water, it is necessary to add the water or buffer solution before adding the olive oil. Otherwise the papain would envelop the lipase in the saponification mixture, thus preventing its action.

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The buffer as well as the amphoteric substances of the papain preparation themselves use up some KOH when in alcoholic solution (12; 20; 22, p. 105; 21, p. 177). Therefore, suitable controls are run with each experiment and this amount of KOH is deducted.

The percentage of cleavage is estimated from the saponification number of the olive oil in the following manner.

100 mg. of papain (Wallerstein) (Table III) reacted with 2.5 gm. of olive oil for 60 minutes at 30°C. in the presence of 4 cc. of 0.25 N acetate buffer (pH 6.0). The amount of alkali used to neutralize the fatty acid after that time was 2.5 cc. of 0.5 N KOH for the experiment and 0.75 cc. of 0.5 N KOH for the control. From the difference of 1.75 cc. of 0.5 N KOH, the saponification is estimated as follows:

$$\frac{1.75 \times 56.11 \times 100}{2 \times 2.5 \times 183} = 10.7 \text{ per cent}$$

*2. Papain Lipase and Hydrogen Ion Concentration.*

We find that the most favorable conditions for the action of the papain lipase are offered in a pH range from about 5.8 to 6.2, if acetate buffer of a normality of 0.1 to 0.5 is used. Phosphate buffers are less satisfactory, and the smallest amount of cleavage is found with citrate buffers, which coincides with Willstätter's findings with castor lipase (21, p. 170).

*3. Lipolytic Action of Different Papain Preparations.*

We find that in different commercial preparations of papain the content of lipase present varies very much. The highest content of lipase is shown by a preparation which we owe to the kindness of Mr. L. Wallerstein; a preparation from Parke, Davis and Company gave the next best results.

*4. Lipolytic Action and Temperature.*

Papain lipase has its temperature optimum at about 35–40°C. At higher temperatures, however, and when the experiments are continued for a longer time, in addition to the injurious influence of the temperature, destruction by the proteolytic enzyme must be taken into consideration.

TABLE I.

*Lipolytic Action and pH.*

*Acetate Buffer.**

(100 mg. papain (Wallerstein), 2.5 gm. olive oil, 4 cc. 0.25 N acetate buffer, 60 min., 30°C.)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| pH | 4.0 | 4.8 | 5.0 | 5.2 | 5.6 | 6.0 | 6.2 | 6.8 |
| Cleavage, *per cent* | 2.2 | 5.6 | 6.6 | 7.7 | 8.8 | 10.7 | 9.8 | 7.9 |

*Phosphate Buffer.*†

(100 mg. papain (Wallerstein), 2.5 gm. olive oil, 4 cc. M/15 phosphate buffer, 60 min., 30°C.)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| pH | 5.3 | 5.6 | 5.9 | 6.2 | 6.4 | 6.6 | 6.8 | 7.3 | 8.0 |
| Cleavage, *per cent* | 5.4 | 6.4 | 8.7 | 8.3 | 8.0 | 7.1 | 6.6 | 6.1 | 5.3 |

*Citrate Buffer.*†

(100 mg. papain (Wallerstein), 2.5 gm. olive oil, 4 cc. 0.1 M citrate buffer, 60 min., 30°C.).

| | | | | | | |
|---|---|---|---|---|---|---|
| pH | 5.0 | 5.3 | 5.6 | 6.0 | 6.3 | 6.7 |
| Cleavage, *per cent* | 1.7 | 2.9 | 3.5 | 5.4 | 5.1 | 4.6 |

*$NH_3$-$NH_4Cl$ Buffer.*‡

(100 mg. papain (Parke, Davis and Company), 2.5 gm. olive oil, 6.0 cc. $H_2O$, 1 cc. 0.5 N $NH_3$-$NH_4Cl$ buffer, pH 8.9, 60 min., 30°C. = 3.5 per cent cleavage).

* Walpole (11).

† According to Sörensen.

‡ According to Michaelis, and Willstätter, Waldschmidt-Leitz, and Memmen, (22), p. 104.

TABLE II.

*Lipolytic Action and Buffer Concentration.*

(100 mg. papain (Wallerstein), 2.5 gm. olive oil, 4 cc. acetate buffer, 60 min., 30°C.)

| pH | Cleavage at various buffer concentrations. | | | |
|---|---|---|---|---|
| | N | 0.5 N | 0.25 N | 0.1 N |
| | *per cent* | *per cent* | *per cent* | *per cent* |
| 5.6 | 7.7 | 7.6 | 8.8 | 9.2 |
| 5.8 | 7.8 | 7.8 | 8.9 | 9.3 |
| 6.0 | 9.8 | 10.0 | 10.7 | 10.3 |
| 6.2 | 9.6 | 9.5 | 9.8 | 10.0 |
| 6.8 | 7.5 | 7.4 | 7.9 | 8.5 |

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*5. Influence of Activators.*

When investigating animal lipases, Willstätter, Waldschmidt-Leitz, and Memmen (22, p. 99) studied the influence of the combined action of several activators[4] (complex or coupled adsorbents) in acid and alkaline media. We found that addition of ovalbumin, HCN, or bile salts has no influence on the lipolytic action of papain lipase, either in an acid or an alkaline medium. Glycerol, however, inhibits in acid and neutral media, but activates at an alkaline pH. $CaCl_2$ activates distinctly at an alkaline reaction. But this activation of papain lipase is diminished by ovalbumin, quite in contrast to Willstätter's findings with pancreatic lipase (22, p. 109). This effect of $CaCl_2$ and ovalbumin can also be seen with a more purified preparation (Table XII).

TABLE III.

*Lipolytic Action of Different Papain Preparations.*

(100 mg. papain, 2.5 gm. olive oil, 4 cc. 0.25 N acetate buffer, pH 6.0, 60 min., 30°C.)

| Wallerstein. | Parke, Davis and Company. | Merck I. | Merck II. | Eimer and Amend |
|---|---|---|---|---|
| *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 10.7 | 8.7 | 6.9 | 3.3 | 2.4 |

TABLE IV.

*Lipolytic Action and Temperature.*

(80 mg. papain (Parke, Davis and Company), 2.5 gm. olive oil, 4 cc. 0.25 N acetate buffer, pH 6.0, 60 min.)

| Temperature. | Cleavage. | | |
|---|---|---|---|
| | 60 min. | 120 min. | 320 min. |
| °C. | *per cent* | *per cent* | *per cent* |
| 25 | 6.1 | 8.7 | 13.5 |
| 30 | 7.6 | 10.8 | 16.7 |
| 35 | 8.7 | 11.9 | 18.0 |
| 40 | 11.2 | 15.2 | 17.7 |
| 50 | 11.7 | 14.1 | 14.8 |
| 60 | 11.2 | 11.7 | 11.9 |

Willstätter states in his work on castor lipase that plant lipase

[4] The effect of individual activators, used separately, was known before.

is not susceptible to activation (21, p. 163). He also finds that an activation of castor lipase by manganese salts, which has been described by Hoyer (7), Tanaka (10), and Falk and Hamlin (4), does not occur under optimal conditions (21, p. 171).

TABLE V.

*Lipase Activation and pH.*

(80 mg. papain (Parke, Davis and Company), 2.5 gm. olive oil, 60 min., 30°C., pH 4.8 and 6.0; 2 cc. 0.5 N acetate buffer + 2 cc. $H_2O$, pH 8.9; 1 cc. $NH_3$-$NH_4Cl$ buffer + 5 cc. $H_2O$. 2 per cent $CaCl_2$ solution, 3 per cent ovalbumin solution.)

| pH | Cleavage. | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Without glycerol. | | | | With 50 per cent glycerol. | | | |
| | Albumin 0 $CaCl_2$ 0 | Albumin 0 $CaCl_2$ 20 mg. | Albumin 15 mg. $CaCl_2$ 0 | Albumin 15 mg. $CaCl_2$ 20 mg. | Albumin 0 $CaCl_2$ 0 | Albumin 0 $CaCl_2$ 20 mg. | Albumin 15 mg. $CaCl_2$ 0 | Albumin 15 mg. $CaCl_2$ 20 mg. |
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| (Acetate buffer.) 4.8 | 4.0 | | | | 2.4 | | | |
| (Acetate buffer.) 6.0 | 7.6 | 7.4 | 7.8 | 7.9 | 4.1 | 4.3 | 4.1 | |
| ($NH_3$-$NH_4Cl$ buffer.) 8.9 | 3.2 | 6.1 | 4.0 | 5.1 | 4.3 | 5.6 | 4.4 | 5.7 |

*Experiment 1.*—80 mg. of papain (Parke, Davis and Company) and 2.5 gm. of olive oil react for 60 minutes at 30°C. with: (*a*) 4 cc. of 0.25 N acetate buffer (pH 6.0); (*b*) 10 mg. of HCN + 4 cc. of 0.25 N acetate buffer (pH adjusted to 6.0); (*c*) 5 cc. of $H_2O$ + 1 cc. of 0.5 N $NH_3$-$NH_4Cl$ buffer (pH 8.9); and (*d*) 5 cc. of $H_2O$ + 10 mg. of HCN + 1 cc. of 0.5 N $NH_3$-$NH_4Cl$ buffer (pH adjusted to 9.2). The amount of cleavage was: (*a*) 7.9 per cent, (*b*) 7.4 per cent, (*c*) 2.9 per cent, and (*d*) = 3.3 per cent. Previous treatment with these buffer solutions for 60 minutes at 30°C. has no influence on the amount of cleavage: (*a*) 7.5 per cent, (*b*) 7.6 per cent, (*c*) 3.2 per cent, and (*d*) 3.0 per cent.

*Experiment 2.*—80 mg. of papain (Parke, Davis and Company), 5 cc. of $H_2O$, and 1 cc. of 0.5 N $NH_3$-$NH_4Cl$ buffer (pH 8.9) were mixed with 2.5 gm.



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of olive oil. Different amounts of the following activators were added and left 60 minutes at 30°C.

| No. | | Amount of cleavage |
|---|---|---|
| | | *per cent* |
| 1 | Without activator. | 3.0 |
| 2 | + 1 mg. $CaCl_2$. | 4.6 |
| 3 | + 2 " " | 4.5 |
| 4 | + 5 " " | 6.0 |
| 5 | + 10 " " | 5.8 |
| 6 | + 20 " " | 6.3 |
| 7 | + 40 " " | 6.1 |
| 8 | + 10 " sodium taurocholate. | 2.9 |
| 9 | + 10 " " glycocholate. | 3.4 |

*6. Kinetics of the Lipolytic Action.*

The lipolytic saponification follows to a certain extent the law of Schütz (Tables VI to XI). The discrepancies that arise upon activation with $CaCl_2$ in an alkaline medium (changing pH) warrant further investigation (Tables VIII, IX, and X).

We have postponed the introduction of a special papain lipase unit (as was done by Willstätter for various other lipases (16, p. 23; 17, p. 237; 22, p. 115; 21, p. 178)) till a lipase has been prepared directly from the latex.

*7. Stability of the Lipase.*

The lipase is relatively stable against the action of the proteolytic enzyme. 100 mg. of papain (Wallerstein) were first treated with: (*a*) 2 cc. of $H_2O$ for 16 hours at 20°C.; (*b*) 2 cc. of $H_2O$ for 16 hours at 37°C.; (*c*) 2 cc. of $H_2O$ for 48 hours at 37°C.; (*d*) 10 mg. of HCN for 16 hours at 20°C.; (*e*) 10 mg. of HCN for 16 hours at 37°C.; and (*f*) 10 mg. of HCN for 48 hours at 37°C.

The amount of cleavage of 2.5 gm. of olive oil (acetate buffer, pH 6.0, 60 minutes, 30°C.) was: (*a*) 9.0 per cent, (*b*) 7.1 per cent, (*c*) 3.2 per cent, (*d*) 8.0 per cent, (*e*) 6.1 per cent, and (*f*) 1.0 per cent.

*8. Purification of the Lipase.*

5 gm. of papain (Parke, Davis and Company) were extracted twice for 10 minute periods with 50 cc. of $H_2O$ at 25°C., centrifuged, and the supernatant clear fluid was decanted. The amount

of cleavage of 6.0 cc. of solution I with 2.5 gm. of olive oil and (*a*) 1 cc. of N acetate buffer (pH 6.0) and (*b*) 1 cc. of N $NH_3$-$NH_4Cl$ buffer (pH 8.9) + 20 mg. of $CaCl_2$ was (*a*) 0.0 per cent

TABLE VI.

*Time Action of Papain Lipase.*

(80 mg. papain (Parke, Davis and Company), 2.5 gm. olive oil, 4 cc. 0.25 N acetate buffer, pH 6.0, 30°C.)

| Time. | Cleavage. | $k$ estimated for | |
|---|---|---|---|
| | | $\frac{x}{t} = k$ | $\frac{x}{\sqrt{t}} = k$ |
| *min.* | *per cent* | | |
| 10 | 2.3 | 0.23 | 0.73 |
| 20 | 4.2 | 0.21 | 0.94 |
| 40 | 6.1 | 0.15 | 0.96 |
| 60 | 7.6 | 0.13 | 0.98 |
| 80 | 8.8 | 0.11 | 0.98 |
| 120 | 10.8 | 0.09 | 0.98 |
| 160 | 12.3 | 0.08 | 0.97 |
| 320 | 16.7 | 0.05 | 0.93 |

TABLE VII.

*Action of Different Amounts of Papain Lipase.*

(Papain (Parke, Davis and Company), 2.5 gm. olive oil, 4 cc. 0.25 N acetate buffer, pH 6.0, 60 min., 30°C.)

| Papain. | Cleavage. | $k$ estimated for | |
|---|---|---|---|
| | | $\frac{x}{e} = k$ | $\frac{x}{\sqrt{e}} = k$ |
| *mg.* | *per cent* | | |
| 20 | 2.0 | 0.10 | 0.45 |
| 40 | 3.9 | 0.097 | 0.62 |
| 80 | 7.6 | 0.095 | 0.85 |
| 100 | 8.7 | 0.087 | 0.87 |
| 160 | 10.9 | 0.068 | 0.86 |
| 200 | 12.2 | 0.061 | 0.86 |
| 320 | 14.8 | 0.046 | 0.83 |

and (*b*) 0.0 per cent. The sediment (0.95 gm.) has a dark brown color and consists of some sand, the remains of cell walls and of a substance which makes a whitish emulsion when stirred with

water. If the sediment is stirred with 20 cc. of $H_2O$ the coarser parts are deposited in a few moments and the emulsion can be decanted. By repeating this treatment, the greater part of the emulsified substance can be obtained. The emulsion is centri-

TABLE VIII.

*Time Action of Activated Papain Lipase.*

(80 mg. papain (Parke, Davis and Company), 2.5 gm. olive oil, 5 cc. $H_2O$, 1 cc. 0.5 N $NH_3$-$NH_4Cl$ buffer, pH 8.9, 20 mg. $CaCl_2$, 30°C.)

| Time. | Cleavage. | $k$ estimated for | |
|---|---|---|---|
| | | $\frac{x}{t} = k$ | $\frac{x}{\sqrt{t}} = k$ |
| *min.* | *per cent* | | |
| 20 | 1.6 | 0.080 | 0.36 |
| 40 | 3.8 | 0.095 | 0.60 |
| 60 | 6.4 | 0.110 | 0.83 |
| 80 | 8.1 | 0.110 | 0.90 |
| 160 | 12.8 | 0.080 | 1.01 |
| 320 | 18.0 | 0.056 | 1.01 |
| 640 | 25.2 | 0.039 | 1.00 |
| 1,280 | 34.2 | 0.027 | 0.96 |

TABLE IX.

*Time Action of Activated Papain Lipase.*

(320 mg. papain (Parke, Davis and Company), 2.5 gm. olive oil, 5 cc. $H_2O$, 1 cc. 0.5 N $NH_3$-$NH_4Cl$ buffer, pH 8.9, 20 mg. $CaCl_2$, 30°C.)

| Time. | Cleavage. | $k$ estimated for | |
|---|---|---|---|
| | | $\frac{x}{t} = k$ | $\frac{x}{\sqrt{t}} = k$ |
| *min.* | *per cent* | | |
| 20 | 8.3 | 0.41 | 0.186 |
| 40 | 12.5 | 0.31 | 0.198 |
| 60 | 15.4 | 0.26 | 0.199 |
| 80 | 17.6 | 0.22 | 0.197 |
| 160 | 24.2 | 0.15 | 0.191 |
| 320 | 33.0 | 0.10 | 0.185 |

fuged and the sediment dried in a vacuum desiccator. It amounted to 0.2 gm. (lipase preparation 3). The usual protein tests were positive. Ash content 35 per cent.

The action of such a purified preparation is shown in Table XII.

TABLE X.

*Action of Different Amounts of Activated Papain Lipase.*

(Papain (Parke, Davis and Company), 2.5 gm. olive oil, 5 cc. $H_2O$, 1 cc. 0.5 N $NH_3$-$NH_4Cl$ buffer, pH 8.9, 20 mg. $CaCl_2$, 60 min., 30°C.)

| Papain. | Cleavage. | $k$ estimated for | |
|---|---|---|---|
| | | $\frac{x}{e} = k$ | $\frac{x}{\sqrt{e}} = k$ |
| *mg.* | *per cent* | | |
| 10 | 1.0 | 0.100 | 0.32 |
| 20 | 2.0 | 0.100 | 0.45 |
| 40 | 3.2 | 0.080 | 0.51 |
| 80 | 6.1 | 0.076 | 0.68 |
| 100 | 7.1 | 0.071 | 0.71 |
| 160 | 10.3 | 0.064 | 0.81 |
| 320 | 15.4 | 0.048 | 0.86 |

TABLE XI.

*Amount of Enzyme and Percentage of Cleavage at Different Times.*

(Papain (Parke, Davis and Company), 2.5 gm. olive oil, 5 cc. $H_2O$, 1 cc. 0.5 N $NH_3$-$NH_4Cl$ buffer, pH 8.9, 20 mg. $CaCl_2$, 30°C.)

| Amount of enzyme. $e$ = 320 mg. | Amount of enzyme × time = $e \times t$ | | | | |
|---|---|---|---|---|---|
| | 20 | 40 | 80 | 160 | 320 |
| 1 (320 mg.) | 8.3 | 12.5 | 17.6 | 24.2 | 33.0 |
| ½ (160 " ) | 9.0 | 11.8 | 18.1 | 25.2 | 32.8 |
| ¼ ( 80 " ) | 8.1 | 12.8 | 18.0 | 25.2 | 34.2 |
| ⅛ ( 40 " ) | 7.7 | 10.9 | 15.4 | 20.5 | |
| 1/16 ( 20 " ) | 6.1 | 8.4 | 12.5 | | |

TABLE XII.

*Action of a Purified Papain Lipase Preparation.*

(20 mg. lipase preparation 3, 2.5 gm. olive oil, 60 min., 30°C.)

| Buffer..... | 4 cc. 0.25 N acetate buffer, pH 6.0. | | 3 cc. $H_2O$, 1 cc. N $NH_3$-$NH_4Cl$ buffer, pH 8.9. | | | |
|---|---|---|---|---|---|---|
| Activator.... | None. | 10 mg. $CaCl_2$. | None. | 10 mg. $CaCl_2$. | 30 mg. ovalbumin. | 10 mg. $CaCl_2$ + 30 mg. ovalbumin. |
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| | 8.6 | 8.9 | 1.6 | 10.9 | 2.6 | 6.1 |

SUMMARY.

Crude papain contains a lipase of considerable activity.

Papain lipase is insoluble in water and has its optimum effect at 35–40°C. at a pH 5.8 to 6.2 in acetate buffer.

Papain lipase follows the law of Schütz to a certain extent.

$CaCl_2$ activates papain lipase in an alkaline medium (changing pH).

Papain lipase differs from castor lipase in its pH optimum and its possible activation by $CaCl_2$ in an alkaline medium (changing pH).

Purification is possible by removing the papain with water.

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23. Willstätter, R., and Waldschmidt-Leitz, E., *Z. physiol. Chem.*, 1923, cxxv, 132.

# ANTHOCYANIN FORMATION IN HELIANTHUS ANNUUS.

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(Received for publication, March 6, 1925.)

Recently the writer has been successful in isolating and identifying the yellow flavonol glucoside, *quercimeritrin*, from the ligulate corollas of the so called orange double chrysanthemum-flowered sunflower. While this pigment is not responsible for the characteristic orange coloration of the flowers, which is undoubtedly caused by one or both of the plastid pigments, carotin and xanthophyll, its presence is of interest because of a bearing on the formation of anthocyanin in the chestnut-red and wine-red varieties.

It will be remembered that the original chestnut-red sunflower[1] was found growing wild in 1910, at Boulder, Colorado, by Mrs. Cockerell. From this single individual there arose through extensive crossing and subsequent segregation probably all of the chestnut-red and wine-red modifications now in cultivation. Most of these reds are plants with heads bearing a single row of colored rays, but at least one red "double-flowered" form has been reported.[2] This red form would correspond to the orange variety mentioned above, which was used for the isolation of quercimeritrin. Although the red varieties so far produced differ from each other in appearance, they do not seem to contain different and distinct anthocyanins. As a matter of fact the chestnut-red color is caused by the presence of the red anthocyanin on an orange background, which is presumably of a plastid origin, while the wine-red modification seems to be due to the same anthocyanin superimposed on a primrose background.

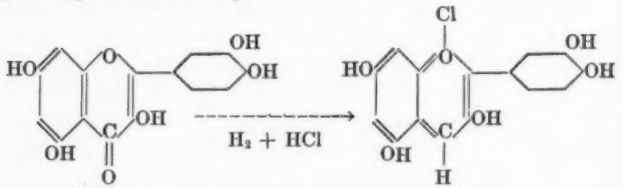
[1] Cockerell, T. D. A., The red sunflower, *Popular Sc. Monthly*, 1912, lxxx, 373; A wine-red sunflower, *Science*, 1913, xxxviii, 312; The story of the red sunflower, *Am. Mus. J.*, 1918, xviii, 38.

[2] Cockerell, T. D. A., Specific and varietal characters in annual sunflowers, *Am. Naturalist*, 1915, xlix, 609.

A number of years ago Miss Wheldale suggested, as a result of extensive cross-breeding experiments,[3] that in many flowers anthocyanin formation in its simplest form could be considered dependent on the presence of a colorless or relatively colorless chromogen of the nature of a flavone[4] glucoside.[5] Later, the theory was extended[6] to indicate that production of anthocyanin probably results from a chemical reaction in the plant between an enzyme and a free flavone pigment or chromogen which becomes oxidized to anthocyanin. The more recent work of Everest[7] and of Willstätter and Mallison[8] tends to prove that the reacting chromogens are in reality glucosides and that the transition from flavone glucoside to anthocyanin is one of reduction and not of oxidation. Thus we find the correspondence between free flavone and anthocyanidin (sugar-free component) as follows:

| Flavone. | | Anthocyanidin (acid-free form). |
|---|---|---|
| Kampherol, $C_{15}H_{10}O_6$ | ⟶ | Pelargonidin, $C_{15}H_{10}O_5$ |
| Quercetin, $C_{15}H_{10}O_7$ | ⟶ | Cyanidin, $C_{15}H_{10}O_6$ |
| Myricetin, $C_{15}H_{10}O_8$ | ⟶ | Delphinidin, $C_{15}H_{10}O_7$ |

The production of anthocyanidin (as chloride) by reduction of flavone in hydrochloric acid solution, by the use of magnesium, may be represented briefly thus:




Quercetin (yellow). Cyanidin chloride (red).

[3] Wheldale, M., The inheritance of flower colour in *Antirrhinum majus*, *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 288.

[4] The term "flavone" is used in its broad sense and includes also the flavonols.

[5] Wheldale, M., On the nature of anthocyanin, *Proc. Cambridge Phil. Soc.*, 1908–10, xv, 137.

[6] Wheldale, M., On the formation of anthocyanin, *J. Genetics*, 1910–11, i, 133.

[7] Everest, A. E., The production of anthocyanins and anthocyanidins, *Proc. Roy. Soc. London, Series B*, 1913–14, lxxxvii, 444.

[8] Willstätter, R., and Mallison, H., Über die Verwandtschaft der Anthocyane und Flavone, *Sitzungsber. k. Akad. Wissensch.*, 1914, 769.

From the modified Wheldale scheme for anthocyanin formation in flowers, which might now be expressed as follows:

Flavone glucoside (Chromogen.) ——————→ Red reduction product (Anthocyanin.)
(Reducing enzyme.)

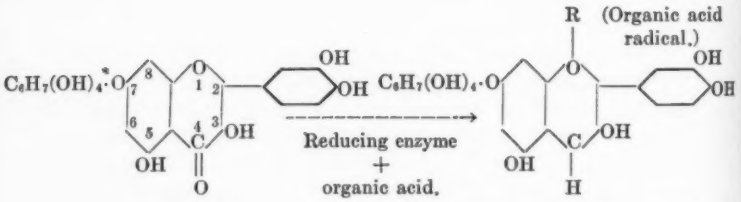
we may still argue that in *Antirrhinum*,[9] for example, there are two factors, *C* and *R*, representing chromogen and enzyme respectively, which may be responsible for the production of anthocyanin. Under this supposition when the crossing of two parents with white flowers produces a progeny whose flowers contain anthocyanin, we would expect the flowers of one parent to contain a chromogen and the flowers of the other an enzyme. *Antirrhinum* is the only case in which the pigments occurring in flowers of known genetical constitution have been investigated chemically. Here we find partial verification of this view since the flowers of one white parent (ivory, except the palate) contain the flavone apigenin, *C*, while the flowers of the other parent, a true white, are totally devoid of chromogen as shown by the fact that the tissues do not yield the characteristic flavone color reaction with ammonia. The presence of a reducing enzyme, *R*, in this parent is merely postulated. Indeed, experimental evidence for the presence of such an enzyme is entirely lacking.

If the foregoing propositions are brought to bear on the case of the red sunflower, we find that here it is equally possible to assume that two factors are responsible for anthocyanin formation, provided we exclude any reference to dilution or pattern formation. In this case we would expect to find certain non-red varieties containing only the chromogen or flavone glucoside and possibly other varieties which might be free of this chromogen, *C*, but possess the ability to produce the enzyme, *R*. It will be difficult, without special breeding, to show the existence of the latter form. We have found in the orange chrysanthemum-flowered sunflower a form which contains the chromogen, but in this particular instance the color happens to be orange because of the presence of plastid pigments, which mask the presence of chromogen, but do not otherwise enter into consideration. Without the plastid pigments, the color would probably be ivory or white, depending

[9] Wheldale, M., The anthocyanin pigments of plants, Cambridge, 1916, 211.

on the amount of flavonol glucoside present. Neither of these relatively colorless forms has been found although the variety *primulinus* approaches them closer in color than any of the other well known forms.

For the purpose of isolating the chromogen from the orange chrysanthemum-flowered form, the corollas from 3,500 heads were collected. The dried material was coarsely ground and extracted with ether to remove plastid pigments and other impurities, after which the extraction was continued with 95 per cent alcohol. From the alcoholic extract 20.5 gm. of pure pigment were obtained. This proved to be a monoglucoside of quercetin known as quercimeritrin which had previously been isolated from cotton flowers. It possesses the formula $C_{21}H_{20}O_{12}$. A more detailed report concerning the isolation and identification of this pigment will be published later. From our knowledge of quercimeritrin it is possible to predict, with the aid of the reduction hypothesis, that the sunflower anthocyanin will very likely prove to be a monoglucoside of cyanidin. The reduction representing its possible formation from quercimeritrin may be expressed as follows:




Quercimeritrin (chromogen).

Oxonium salt of cyanidin glucoside (anthocyanin).

* According to A. G. Perkin, the place of sugar attachment in quercimeritrin is represented by either 5 or 7.

# THE DETERMINATION OF SMALL AMOUNTS OF PROTEIN NITROGEN.

By EDNA RUTH MAIN and ARTHUR P. LOCKE.*

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(Received for publication, March 20, 1925.)

The investigations of one of us toward the isolation and examination of an "immune" hemolysin[1] depend upon the determination, at each stage of the fractionation, of the amount of protein associated with one hemolytic unit. Preparations of rabbit anti-sheep hemolysin are being made of such potency that one hemolytic unit is associated with but $6 \times 10^{-8}$ gm. of nitrogen. The conservation of this material is requiring an extension of the simpler existing methods for the recognition and estimation of very small amounts of the proteins.

The excellent method of Folin and Denis[2] is adapted to the easy estimation of quantities of protein nitrogen with a precision extending to $10^{-6}$ to $10^{-7}$ gm., when sufficient material is available to give a final concentration of $2 \times 10^{-4}$ gm. of nitrogen per 100 cc. of the Nesslerized solution. This latter limitation is imposed by the Duboscq colorimeter. For smaller amounts of available material, producing less than $10^{-4}$ gm. of protein nitrogen, we have found it advantageous to measure the colors of the final Nesslerized solutions with a spectrometer. The Keuffel and Esser color analyser was used.

In this instrument, parallel beams of monochromatic light reach the eye; one has passed through the Nesslerized solution and the other through distilled water. The intensity of the monochromatic beam from the distilled water can be diminished to a value exactly equal to that coming from the unknown solution. This

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[1] Locke, A., and Hirsch, E. F., *J. Infect. Dis.*, 1924, xxxv, 519.

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

transmission value is recorded and subtracted from a value obtained when the Nesslerized sample is replaced by a Nesslerized blank solution. The difference represents light absorbed by the mercury-ammonium complex and is proportional to the concentration of ammonia in the sample.

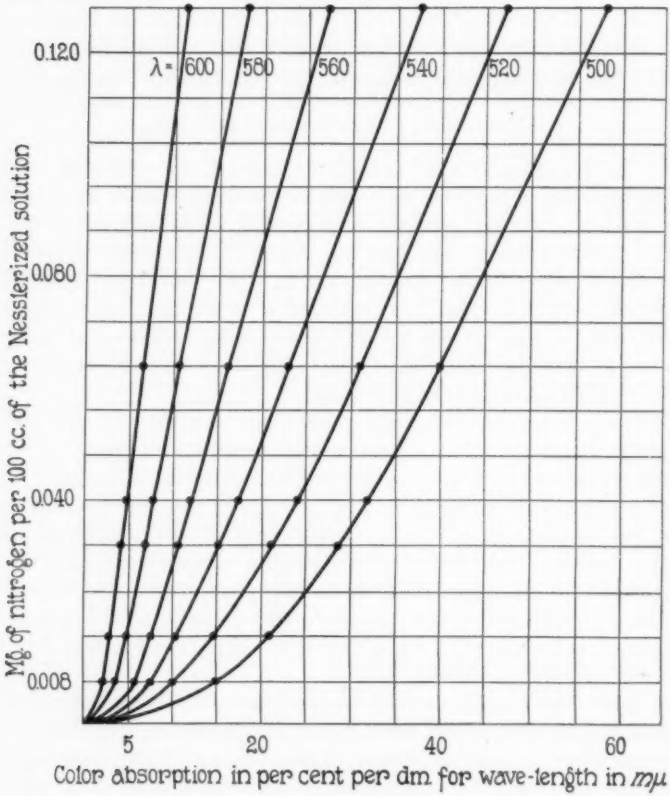


FIG. 1. Reference curves for the conversion of the color absorption values of a Nesslerized solution to nitrogen content.

The light absorption of a series of Nesslerized solutions of ammonium sulfate was determined at wave-lengths: 500, 520, 540, 560, 580, and 600$m\mu$. The values are recorded in Fig. 1

and Table I. The absolute color production is influenced by many experimental conditions, but color values are sufficiently constant for Nesslerized solutions containing less than 0.05 mg. of nitrogen per 100 cc. that the curves may be used for reference.

Digestion and Nesslerization of the sample are carried out precisely as suggested by Folin and Denis.[2] It is not advisable to use a sample containing more than 0.02 to 0.03 mg. of nitrogen, and the digestion mixture should be diluted to 6 cc. before evaporation and digestion are commenced. Dehydration of the phosphoric acid is to be avoided.

TABLE I.

*Conversion of Color Absorption to Mg. of Nitrogen.*

| $N_2$ | Color absorption in per cent per dm. for wave-length in $m\mu$. | | | | | |
|---|---|---|---|---|---|---|
| | 500 | 520 | 540 | 560 | 580 | 600 |
| *mg.* | | | | | | |
| 0.0025* | 8 | 6 | 4 | 3.5 | 2 | 1.5 |
| 0.005* | 12 | 8.5 | 6.5 | 5 | 3.3 | 2 |
| 0.008 | 15 | 10 | 8 | 6 | 4 | 2.3 |
| 0.012* | 18 | 13 | 9 | 6.7 | 4.5 | 2.7 |
| 0.016 | 20.3 | 14.7 | 10 | 7.5 | 5 | 3 |
| 0.024* | 24.7 | 18 | 12.5 | 9 | 6 | 3.5 |
| 0.032 | 28 | 21 | 15 | 10.7 | 6.7 | 4 |
| 0.04* | 32 | 24 | 17 | 12 | 7.5 | 4.5 |
| 0.064 | 40 | 31 | 23 | 16 | 10.3 | 6.3 |
| 0.128 | 58 | 47 | 37 | 27 | 18 | 11 |

* Interpolated value. The color variation is practically linear between the values indicated.

The Nesslerized solution is centrifugated for 10 minutes, decanted into a 10 cm. color tube, and the color absorption read at once as indicated above. The absorption value is converted to a nitrogen value by consultation of Table I.

Table II is a collection of the results of analyses made by this spectrometric modification of the method of Folin and Denis. The method may be used with considerable success for samples containing only 0.01 mg. of nitrogen, and with fair success for samples containing 0.005 mg. of nitrogen. The experimental

TABLE II.

*Determinations of Protein Nitrogen by an Extension of the Method of Folin and Denis.*

| Sample. | Volume. | Nitrogen per 100 cc. of the Nesslerized solution, from color absorption readings (see Table I) at the following wave-lengths. | | | | | | | Nitrogen per cc. of sample. |
|---|---|---|---|---|---|---|---|---|---|
| | | 500 | 520 | 540 | 560 | 580 | 600 | Mean. | |
| | | | | | | | | | *mg.* |
| Human plasma 1, 1/1,000 dilution... | 1 | 0.021 | 0.019 | 0.022 | 0.019 | | | 0.020 ± 1 | |
| | 1 | 0.023 | 0.022 | 0.022 | 0.019 | 0.024 | | 0.022 ± 1 | 0.0105 ± 5 |
| | 0.5 | 0.008 | 0.011 | 0.012 | 0.011 | 0.008 | | 0.010 ± 1 | |
| | 0.5 | 0.011 | 0.011 | 0.008 | 0.008 | | | 0.010 ± 1 | 0.0100 ± 10 |
| Folin method..... | 20 | | | | | | | 0.425 ± 4 | |
| | 20 | | | | | | | 0.423 ± 4 | 0.0106 ± 1 |
| Human plasma 2, 1/1,000 dilution... | 1 | 0.017 | 0.022 | 0.022 | 0.019 | | | 0.020 ± 2 | |
| | 1 | 0.017 | 0.022 | 0.026 | 0.019 | | | 0.021 ± 3 | 0.102 ± 5 |
| | 0.5 | 0.010 | 0.011 | 0.009 | 0.014 | | | 0.011 ± 1 | |
| | 0.5 | 0.010 | 0.012 | 0.012 | | 0.008 | | 0.011 ± 1 | 0.0110 ± 10 |
| Folin method..... | 20 | | | | | | | 0.400 ± 4 | |
| | 20 | | | | | | | 0.398 ± 4 | 0.0100 ± 1 |
| Human plasma 3, 1/1,000 dilution... | 2 | 0.052 | 0.054 | 0.044 | 0.040 | 0.044 | 0.060 | 0.049 ± 3 | |
| | 2 | 0.049 | 0.054 | 0.048 | 0.052 | 0.044 | 0.046 | 0.049 ± 2 | 0.0122 ± 5 |
| | 1 | 0.027 | 0.027 | 0.026 | 0.024 | 0.024 | | 0.026 ± 1 | |
| | 1 | 0.021 | 0.024 | 0.026 | 0.024 | 0.024 | 0.032 | 0.025 ± 1 | 0.0127 ± 5 |
| Folin method..... | 20 | | | | | | | 0.490 ± 5 | |
| | 20 | | | | | | | 0.487 ± 5 | 0.0122 ± 1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Folin method | 20 | | | | | | | 0.490 ± 5 | |
| | 20 | | | | | | | 0.487 ± 5 | 0.0122 ± 1 |
| Rabbit serum 4, 1/1,000 dilution | 1 | 0.023 | 0.022 | 0.019 | 0.019 | | | 0.021 ± 1 | |
| | 1 | 0.021 | 0.022 | 0.022 | 0.019 | 0.024 | 0.016 | 0.021 ± 1 | 0.0105 ± 5 |
| | 0.5 | 0.008 | 0.012 | 0.012 | 0.014 | | 0.005 | 0.0105 ± 10 | |
| | 0.5 | 0.010 | 0.011 | 0.008 | 0.008 | | | 0.0100 ± 10 | 0.0103 ± 10 |
| Rabbit serum 5, 1/1,000 dilution | 2 | 0.036 | 0.044 | 0.040 | 0.040 | 0.044 | | 0.041 ± 2 | |
| | 2 | 0.046 | 0.040 | 0.040 | 0.034 | 0.035 | | 0.039 ± 2 | 0.0100 ± 5 |
| | 1 | 0.021 | 0.019 | 0.022 | 0.019 | 0.016 | 0.016 | 0.019 ± 1 | 0.0095 ± 5 |
| Folin method | 20 | | | | | | | 0.388 ± 4 | |
| | 20 | | | | | | | 0.388 ± 4 | 0.0097 ± 1 |
| Rabbit serum 6, 1/1,000 dilution | 2 | 0.043 | 0.040 | 0.040 | 0.040 | 0.035 | 0.032 | 0.039 ± 2 | |
| | 2 | 0.043 | 0.040 | 0.040 | 0.034 | 0.044 | 0.032 | 0.039 ± 2 | 0.0098 ± 5 |
| Folin method | 20 | | | | | | | 0.392 ± 4 | |
| | 20 | | | | | | | 0.404 ± 4 | 0.0100 ± 1 |

errors have not permitted the use of smaller samples excepting where deviations of 20 to 80 per cent are of minor importance.

We wish to acknowledge our appreciation of the assistance in this investigation of Dr. Edwin F. Hirsch.

## CONCLUSION.

The writers propose a spectrometric extension of the method of Folin and Denis for the estimation of small quantities of ammonia nitrogen. The extended method permits the use of samples containing as little as 0.005 mg. of protein nitrogen with fair accuracy and with great facility. The field of the method lies in those investigations where only minute quantities of nitrogenous material are available for analysis.

XUM

# THE EFFECT OF ULTRA-VIOLET IRRADIATION ON THE STATE OF THE SERUM CALCIUM.

By ALAN R. MORITZ.

*(From the Department of Pathology, Hanna Research Fellowship, School of Medicine of Western Reserve University, Cleveland.)*

(Received for publication, February 24, 1925.)

## INTRODUCTION.

Disorders of calcium metabolism are not always indicated by changes in the calcium content of body fluids or excretions. It has been long established that in the face of a normal calcium intake the individual may have a low blood calcium with symptoms relievable by the intravenous administration of one of the calcium salts. Again there may be defective utilization of calcium by the body even when the amount of that element in the food and blood is normal. This is best exemplified by low phosphorus rickets.

A study of the state of calcium in the serum may throw some light on the means by which the assimilation and utilization of calcium is accomplished. In 1911 Rona and Takahashi showed that the calcium of the blood differed from the other salts in that about 25 per cent of it was in colloidal combination and would not pass a membrane impervious to colloids. Cushny (1919) corroborated their findings and suggested that the peculiar distribution of the blood calcium might account for the excretion in part by the intestinal mucosa. These observations have stimulated a new line of investigation, *viz.* whether the state of the serum calcium is related to its availability for body needs; and if this be the case, by what means we may alter the state of the serum calcium. In this investigation some phases of this problem were studied.

*Determination of Diffusible Calcium.*—Three different methods have been employed in the past for the determination of the

diffusible calcium of the blood serum: compensation dialysis (Rona and Takahashi; Cruickshank; von Meysenbug and coworkers); simple dialysis against calcium-free physiological salt solution (Clark); and ultrafiltration through a dry semipermeable membrane (Cushny; Neuhausen and Pincus).

Dialysis to determine the actual percentage of the serum calcium is unsatisfactory because of the existence of the Donnan equilibrium on the sides of the membrane. Bayliss demonstrated that there may be a permanent inequality of the distribution of a freely diffusible salt on the two sides of a semipermeable membrane. If a solution of Congo red, which is a compound of a non-diffusible colloid acid with sodium, be placed together with sodium chloride on one side of a membrane and dialysis be allowed to proceed, it will be found that a larger percentage of the sodium chloride is present on the outside than on the inside of the membrane. While it is very true that a permanent equilibrium is established, the distribution of the diffusible salt varies with the amount of the sodium-containing colloid on one side of the membrane. It is probable that the calcium-containing colloid of the serum acts in a manner similar to Congo red.

While ultrafiltration, as used by Cushny and Neuhausen and Pincus, was satisfactory for their needs, we have found it cumbersome and inaccurate when dealing with small quantities of serum. The drying of the membrane and a deposit of a film of serum may alter the results but little when dealing with such large quantities of serum as they used, but these factors introduce an error of almost 50 per cent when 2 cc. samples of serum are tested.

In this investigation a negative pressure filtration method, to be described, was employed for the determination of the diffusible calcium in small quantities of serum. By this method it was found that all of the diffusible calcium was present in the filtrate at the end of 4 hours and that there was no liberation of the colloid-bound calcium even up to 20 hours of filtration. The special advantage of the method is that small quantities of serum can be investigated.

### *Method.*

Separation of the diffusible from the colloidal calcium is accomplished by placing serum in a collodion sac which is immersed

in distilled water and subjected to a negative pressure of 150 mm. of Hg.

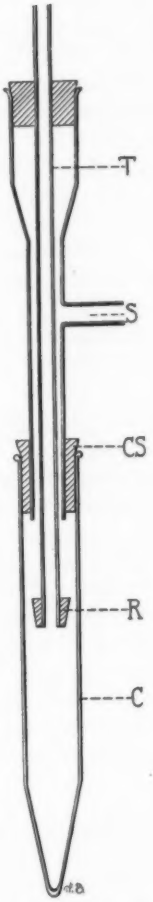


FIG. 1. Diagram of ultrafilter.

The apparatus shown in Fig. 1 is prepared from glass tubing. (*C*) is an ordinary 15 cc. centrifuge tube containing 5 cc. of $H_2O$.

The outside diameter of the tip must not exceed 5 mm. since the calcium determination is to be made in this tube. Tube arm (*S*) is connected with negative pressure and a mercury manometer. 2 cc. of serum obtained by centrifugalization of clotted blood are pipetted into a collodion sac which had been rinsed with saline solution and drained as completely as possible without too much drying. The sac is then tied to a small perforated rubber stopper (*R*). The rubber stopper (*CS*) is now fitted into the mouth of the centrifuge tube, negative pressure of 150 mm. of Hg is applied, and the serum level kept adjusted to the filtrate level by means of tube (*T*). 5 hours is the routine duration of filtration for reasons that will be evident later. It is important that immediately upon immersing the sac in water negative pressure be applied and continued until the sac is removed. 1 cc. of a saturated solution of ammonium oxalate is added directly to the filtrate and the calcium determination is made according to the method described by Kramer and Tisdall.

*Preparation of Collodion Sacs.*—10 gm. of Parlodion and 5 cc. of olive oil are added to 50 cc. of absolute ethyl alcohol and allowed to remain overnight in the incubator at 37°C. In the morning 50 cc. of ether are added and the solution is thoroughly mixed. Sacs are made in glass tubes 1 cm. in diameter by 7 cm. in length. The tube is filled with collodion, centrifuged at about 1,500 R.P.M. for 2 minutes, and drained. To obtain a bag of the desired thickness this procedure is immediately repeated and the sac allowed to dry for 10 minutes. The tube is then immersed in water to remove the sac which is stored in saline solution in the refrigerator. Such sacs may be used repeatedly and will keep for months. These sacs will withstand a negative pressure of 200 mm. of Hg for hours without breaking or allowing any of the protein to pass.

*Experiment 1.*—A rabbit was etherized lightly, bled to death from the femoral artery, and the serum separated from the clotted blood by centrifugalization. Twelve 2 cc. samples of the serum were filtered for varying periods (see Table I). All filtrates were found to be negative for proteins or protein-split products by the biuret and Heller's ring test.

Table I indicates that all the diffusible calcium passes the membrane within the first 4 hours of filtration and that up to at least

20 hours there is no evidence of a split of the calcium-colloid combination.

*Experiment 2.*—Fresh rabbit serum was used and determinations were made of the percentage of diffusible calcium in this serum at definite

TABLE I.

Total amount of calcium in 2 cc. of serum = 0.247 mg.

| Duration of filtration. | Amount of Ca in filtrate. | Percentage of diffusible Ca of serum. |
|---|---|---|
| *hrs.* | *mg.* | *per cent* |
| 1 | 0.092 | 37 |
| 2 | 0.099 | 40 |
| 3 | 0.116 | 47 |
| 4 | 0.132 | 53 |
| 5 | 0.133 | 54 |
| 6 | 0.127 | 51 |
| 8 | 0.129 | 52 |
| 10 | 0.133 | 54 |
| 12 | 0.134 | 54 |
| 14 | 0.124 | 50 |
| 16 | 0.135 | 55 |
| 20 | 0.135 | 55 |

TABLE II.

Total amount of calcium in 2 cc. of serum = 0.243 mg.

| Serum. | Amount of Ca in filtrate. | Percentage of diffusible Ca of serum. |
|---|---|---|
| | *mg.* | *per cent* |
| Fresh serum | 0.140 | 58 |
| | 0.137 | 56 |
| Refrigerator (24 hrs.) | 0.138 | 57 |
| | 0.142 | 58 |
| " (48 " ) | 0.143 | 59 |
| | 0.131 | 54 |
| Room temperature (24 hrs.) | 0.151 | 62 |
| | 0.156 | 64 |
| " " (48 " ) | 0.167 | 69 |
| | 0.174 | 72 |

intervals after bleeding; one portion being kept at room temperature (15–20°C.) and the other kept in the refrigerator at 5°C. All specimens were filtered for 5 hours. The results are shown in Table II.

XUM

It is seen that there is no significant alteration in percentage of diffusible calcium up to 48 hours in the refrigerator, but there is a distinct progressive increase in the percentage of diffusible calcium of serum standing at room temperature.

*Experiment 3.*—It was deemed advisable finally to test this method with sera known to have marked differences in the percentage of diffusible calcium. This was done by anesthetizing a rabbit with urethane and placing a cannula in the femoral artery. 4 cc. of 3 per cent $CaCl_2$ were injected into the ear vein. Immediately before and after the injection of $CaCl_2$ and at 20 minute intervals for 2 hours thereafter samples of blood were collected and determinations of diffusible calcium made. The diffusible calcium varied from 45 to 68 per cent, thus showing that differences in relative amounts of diffusible and non-diffusible calcium of the serum can be detected by the method.

### *Effect of Ultra-Violet Irradiation on the State of the Serum Calcium.*

A rachitic animal may have a normal amount of blood calcium and if that animal be exposed each day to ultra-violet light for a given number of days, calcification is stimulated and the rickets cured. It becomes of interest then to observe the effect of ultra-violet irradiation on the state of the serum calcium since the change is not necessarily in the amount of calcium in the serum.

The source of the ultra-violet light in the following experiments was a Cooper-Hewitt Uviarc Laboratory Outfit, operating on 220 volts and 4.5 amperes, direct current.

*Experiment 4.*—Normal rabbit, sheep, and human sera were exposed for varying lengths of time both in quartz vessels and in shallow uncovered glass containers. Controls were made in all instances with serum protected from the ultra-violet light by thick glass. The temperature of the serum was not allowed to rise above 20°C. Table III is the result of irradiating rabbit serum and is representative of five experiments on the irradiation of rabbit serum *in vitro* and parallels the results of ten experiments on sera of different animals.

Table III indicates that ultra-violet irradiation of serum *in vitro* produces no significant change in the percentage of the diffusible calcium. The variations recorded are well within the error of the method.

*Experiment 5.*—Normal adult rabbits were shaved from the ears to the rump on the day preceding the experiment. They were exposed to the ultra-violet light at a distance of 70 cm. from the quartz tube and the temperature of the animal box kept between 15° and 20°C. Five animals were used and a sample of blood was taken immediately before and after irradiation.

TABLE III.

| Serum. | Amount of Ca in filtrate. | Percentage of diffusible Ca of serum. |
|---|---|---|
| Amount of calcium in 2 cc. of rabbit serum = 0.265 mg. | | |
| | *mg.* | *per cent* |
| Non-irradiated serum | 0.146 | 55 |
| Irradiated (2 hrs.) | 0.141 | 53 |
| " (4 " ) | 0.139 | 52 |
| Amount of calcium in 2 cc. of rabbit serum = 0.285 mg. | | |
| Non-irradiated serum | 0.133 | 47 |
| Irradiated (2 hrs.) | 0.136 | 48 |
| " (4 " ) | 0.132 | 46 |

TABLE IV.

| Animal No. | Before irradiation. | | | Time of UV exposure. | After irradiation. | | |
|---|---|---|---|---|---|---|---|
| | Amount of Ca in 2 cc. of serum. | Amount of Ca in filtrate. | Percentage of diffusible Ca. | | Amount of Ca in 2 cc. of serum. | Amount of Ca in filtrate. | Percentage of diffusible Ca. |
| | *mg.* | *mg.* | *per cent* | *hrs.* | *mg.* | *mg.* | *per cent* |
| 1 | 0.268 | 0.157 | 59 | 3 | 0.252 | 0.141 | 56 |
| 2 | 0.299 | 0.162 | 54 | 3 | 0.305 | 0.162 | 53 |
| 3 | 0.315 | 0.129 | 41 | 3 | 0.309 | 0.131 | 42 |
| 4 | 0.239 | 0.127 | 53 | 2 | 0.228 | 0.129 | 57 |
| 5 | 0.222 | 0.119 | 54 | 2 | 0.214 | 0.103 | 48 |

Table IV shows that there is no significant alteration in the percentage of the diffusible calcium of the serum of animals immediately following irradiation.

*Experiment 6.*—Two adult rabbits were irradiated for 1 hour every other day beginning Jan. 9 and ending Jan. 15. Diffusible calcium determinations on the serum were made before and after irradiation on the 1st day, and immediately following each irradiation thereafter. The rabbits are designated as Nos. 1 and 2.

Table V indicates that intermittent irradiation over several days' time produces no significant change in the percentage of the diffusible calcium of the serum.

TABLE V.

| Date. | Animal No. | Amount of Ca in 2 cc. of serum. | Amount of Ca in filtrate. | Percentage of diffusible Ca. |
|---|---|---|---|---|
| | | Before irradiation. | | |
| | | *mg.* | *mg.* | *per cent* |
| Jan. 9 | 1 | 0.261 | 0.151 | 58 |
| | 2 | 0.234 | 0.138 | 59 |
| | | After irradiation. | | |
| Jan. 9 | 1 | 0.252 | 0.156 | 62 |
| | 2 | 0.241 | 0.136 | 56 |
| " 11 | 1 | 0.273 | 0.153 | 56 |
| | 2 | 0.237 | 0.145 | 61 |
| " 13 | 1 | 0.268 | 0.143 | 53 |
| " 15 | 1 | 0.244 | 0.165 | 68 |
| | 2 | 0.232 | 0.143 | 61 |

## SUMMARY AND CONCLUSION.

A method for the separation of the diffusible calcium of the blood serum is described which consists of applying a negative pressure of 150 mm. of Hg on the outside of a collodion sac containing serum and immersed in water.

2 cc. of serum are required and all of the diffusible calcium is obtained in the filtrate within 5 hours. Filtration for 20 hours failed to show any further liberation of the colloid-bound calcium. Results by this method show a variability of about 4 per cent and the method is sensitive to artificially induced changes in the percentage of the diffusible calcium of serum.

No changes in the total calcium nor in the percentage of diffusible calcium of the blood serum of normal rabbits were observed following ultra-violet irradiation *in vitro* or *in vivo*.

Dr. Harry Goldblatt's kindly criticisms and helpful suggestions are gratefully acknowledged.

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# OBSERVATIONS ON BLOOD PHOSPHATES AS RELATED TO CARBOHYDRATE METABOLISM.

By A. BOLLIGER and F. W. HARTMAN.

*(From the Laboratories of the Henry Ford Hospital, Detroit.)*

(Received for publication, January 17, 1925.)

The observations recorded below bring out the following points: the diabetic state is demonstrated early in the curve of blood phosphates; blood phosphates are depressed during carbohydrate metabolism only when insulin is available; the complete absence of pancreatic hormone is shown by the blood phosphate level which remains unaffected as a straight line during carbohydrate metabolism; as concerns phosphate metabolism, pituitrin is a direct antagonist of insulin; and carbohydrates and phosphates are best utilized when there is an excess of each in the circulation.

Fiske (1921) (1) was perhaps the first to call attention to the decreased output of urinary phosphates after sugar ingestion. On the other hand, Blatherwick, Bell, and Hill (1924) (2) state, as regards inorganic phosphates of the blood serum during the sugar tolerance test "there sometimes was an increase, a decrease, or no change." Harrop and Benedict (1924) (3) and Perlzweig, Latham, and Keefer (1923) (4) demonstrated a marked fall in the inorganic phosphates of the blood with lessened phosphate excretion in the urine after glucose ingestion. Sokhey and Allan (1924) (5) pointed out that the depression in urinary phosphates after glucose ingestion was almost as great as that after insulin administration. The consistent action of insulin in depressing the inorganic phosphates of the blood was first observed by Wigglesworth, Woodrow, Smith, and Winter (1923) (6) and Winter and Smith (1924) (7) in the normal animal; in both normal and diabetic individuals by Blatherwick, Bell, and Hill (1924) (2), Harrop and Benedict (1924) (3), and Perlzweig, Latham, and Keefer (1923) (4). The last named authors state without further comment that "With diabetic subjects it was frequently ob-



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served that the ingestion of glucose or the injection of insulin did not cause a retention of phosphate especially when the blood sugar curves and the respiratory exchange indicated that there was no increased catabolism of carbohydrate."

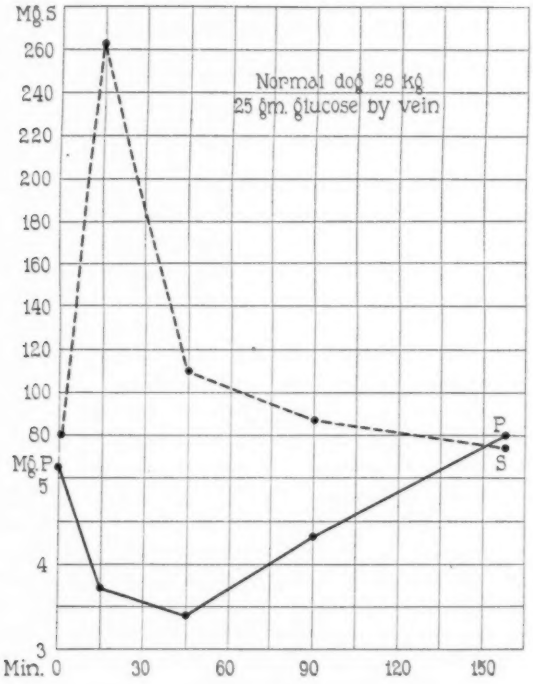


CHART 1.

*Methods.*

For blood sugar determinations Folin and Wu (8) were followed. For the inorganic blood phosphates Benedict's (9) new method was used and in our hands was more accurate and more economical as regards time and material than any of the other methods tried. Blood counts, hemoglobin estimations, and hematocrit readings on blood samples in no instance showed variations which would appreciably alter the chemical observations.

EXPERIMENTAL.

The variation in the blood phosphates of the diabetic individual after insulin and glucose ingestion (Blatherwick, Bell, and Hill (2), Harrop and Benedict (3), Perlzweig, Latham, and Keefer (4)), we felt could be studied advantageously in dogs after the removal of varying amounts of pancreas, and further, that the one extreme should be represented by the blood phosphates as determined during the sugar tolerance test of the normal dog and the other extreme represented during the sugar tolerance test of the completely depancreatized animal (Chart 1). As noted by Harrop and Benedict, the deepest point in the phosphate curve

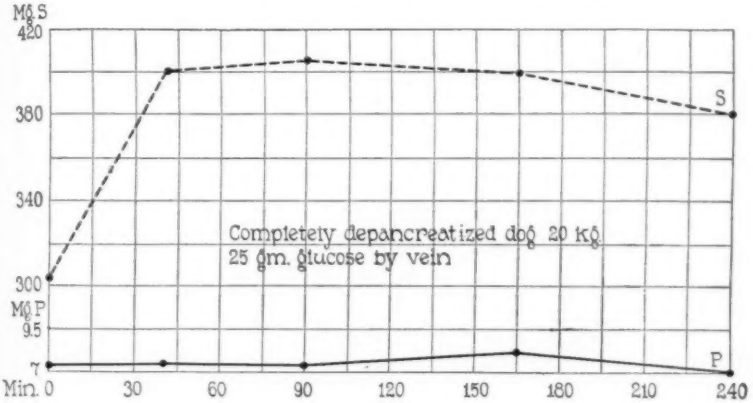


CHART 2.

lags well behind the highest point in the sugar curve. The lowest point in the curve of inorganic phosphates averaged 40 minutes after the glucose injection in normal dogs and individuals in this series of experiments. The completely depancreatized animal, proven by autopsy and microscopic examination of possible remnants, is represented in Charts 2 and 3. In these experiments we have conditions mentioned by Perlzweig, Latham, and Keefer; that is, the sugar curve indicates that there is no catabolism of carbohydrates, and in the straight line phosphorus curve we find the explanation for the lack of phosphate retention noted by them; that is, when there is no catabolism of sugar there is no demand for

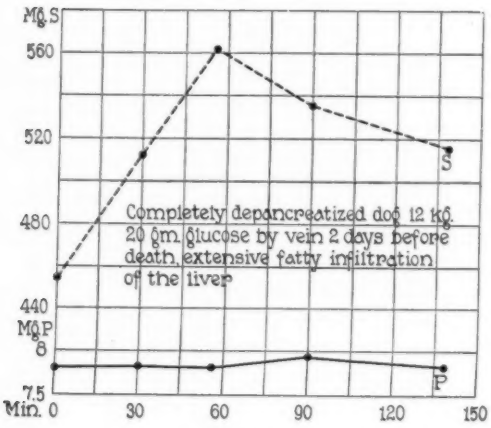


CHART 3.

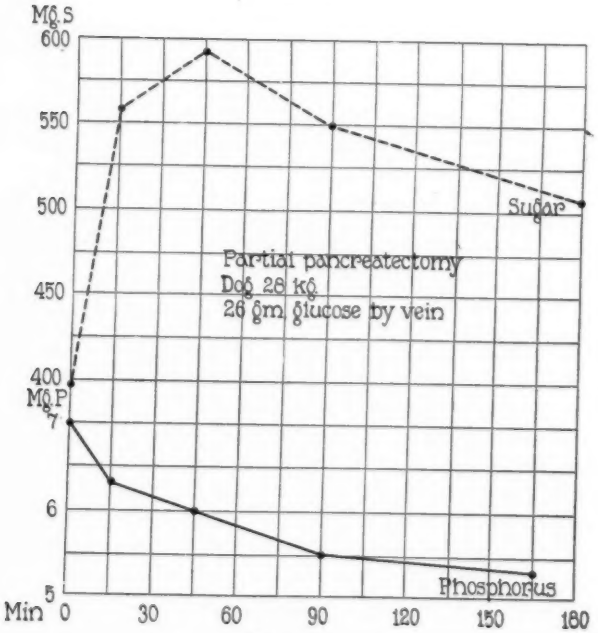


CHART 4.

inorganic phosphates. This finding is confirmed and further emphasized in the curves of partially depancreatized dogs and those of diabetic patients (Chart 4). 95 per cent of the pancreas has been removed as shown by autopsy and actual weight of the remnants. The lowest point in the phosphate curve is reached

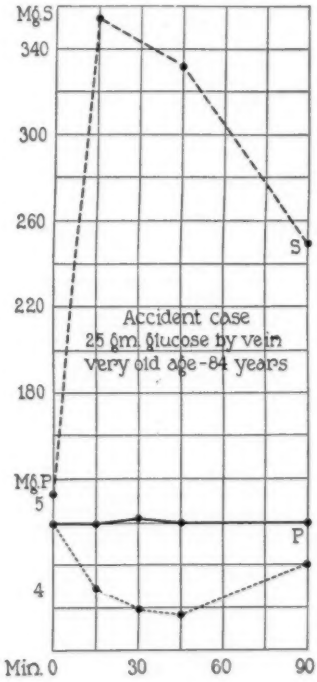


CHART 5.

at the end of the 3 hour period. The influence of a very small amount of pancreas is illustrated on the sugar curve, but especially on the phosphate curve whose slow, moderate, and much prolonged depression demonstrates the demand for phosphates. Typical examples of clinical cases observed are shown in Charts 5, 6, 7, and 8.

The second group of experiments, that is, the effect of insulin

upon blood phosphates, has already received much attention—Wigglesworth, Woodrow, Smith, and Winter (6) and Winter and Smith (7). In the light of data submitted above it would seem that whenever the blood sugar is reduced under the influence of insulin there must be a corresponding depression of the blood phosphates (Chart 9). On the other hand, the relative insufficiency of insulin is well illustrated in two experiments on Chart

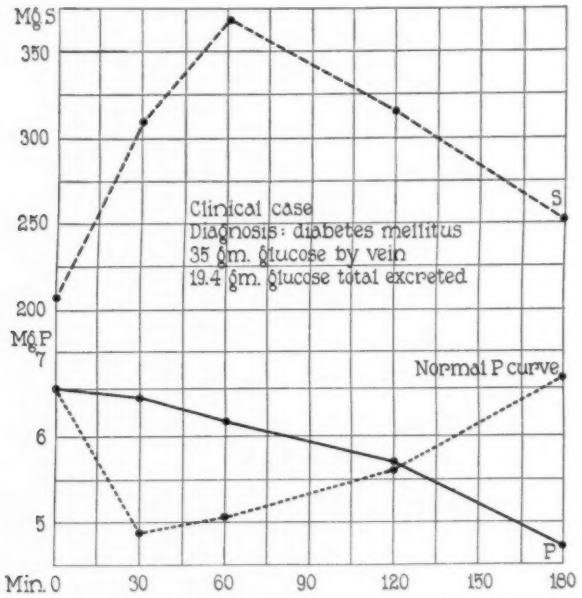


CHART 6.

10. The only exception is found when a very large dose of insulin or repeated smaller doses of insulin are administered producing extreme hypoglycemia below 40 mg. per 100 cc. In this case the inorganic phosphates of the blood are depressed no further, but, on the other hand, start a progressive rise which lasts throughout the hypoglycemic symptoms manifested by muscular twitching and incoordination (Chart 11). Similarly, there is no demand for inorganic phosphates during the hypoglycemic state treated with

glucose administration until the blood sugar level approaches normal (Chart 12).

*Medullary Puncture (Piqûre).*—The hyperglycemia resulting from puncture of the so called glycogenic or diabetic center in the floor of the fourth ventricle is accompanied by a marked and prolonged depression of blood phosphates similar to that seen in the mildest grade of diabetes (Chart 13).

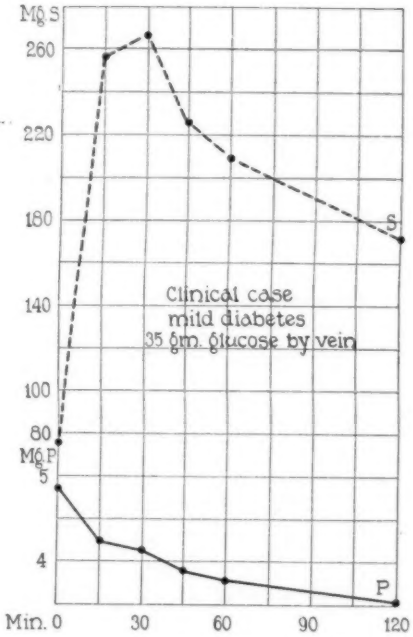


CHART 7.

*Administration of Adrenalin.*—The effect of the administration of adrenalin on blood and urine phosphates has been studied in the normal by Perlzweig, Latham, and Keefer (4), Winter and Smith (7), and Allan, Dickson, and Markowitz (10). These authors are in practical agreement as to the retention of urine phosphates and the depression of the blood phosphates. Further experiments on normal dogs show phosphate curves similar to

those after glucose and after insulin, but they are greatly exaggerated, the depression being sharper, more rapid, and of longer duration (Chart 14). This exaggeration of the phosphate depression suggests at once a rapid conversion of glycogen to glucose in the liver and a greater demand for phosphates than is anticipated

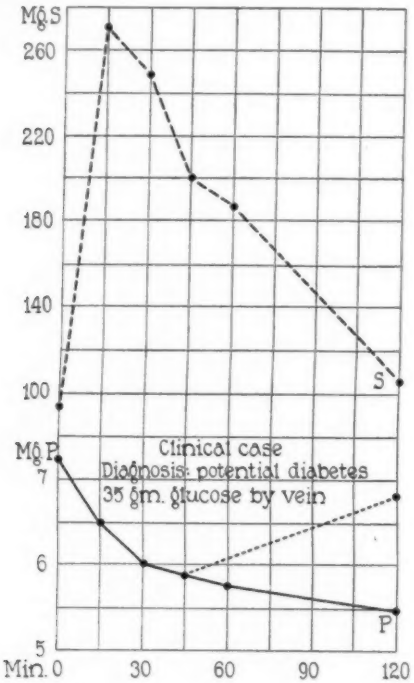


CHART 8.

from the hyperglycemia. This inference, however, is shown to be untenable by administration of similar doses to completely depancreatized dogs of similar size and weight with practically no disturbance in the inorganic phosphate content of the blood. This finding on the blood phosphates is in agreement with the finding of Allan, Dickson, and Markowitz as to phosphate excre-

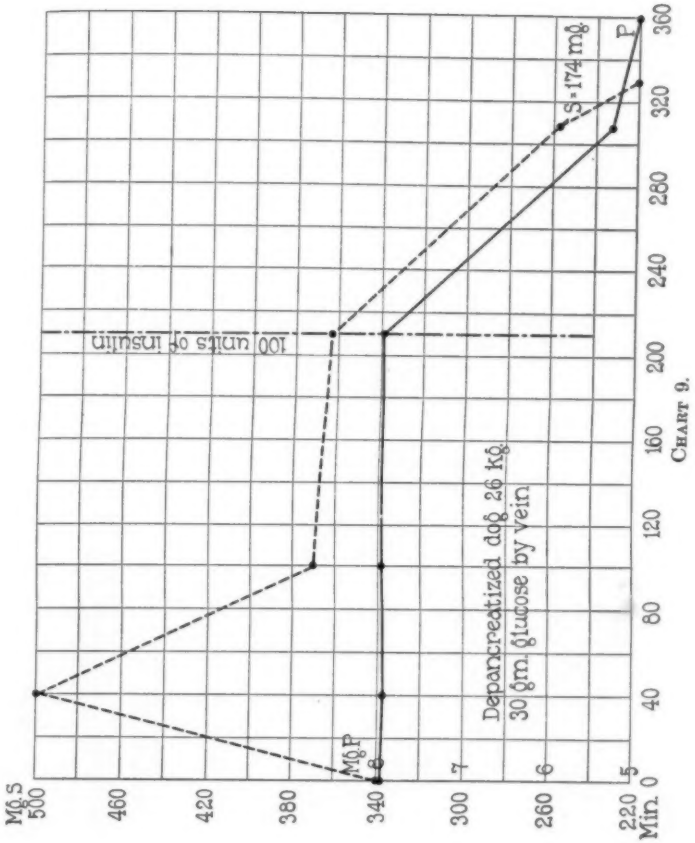
100 units of insulin
Depancreatized dog 26 Kg.
30 gm. glucose by vein
S=174 mg.
P
M.S
M.P
Min. 0
5
6
7
8
220
260
300
340
380
420
460
500
40
80
120
160
200
240
280
320
360

Chart 9.

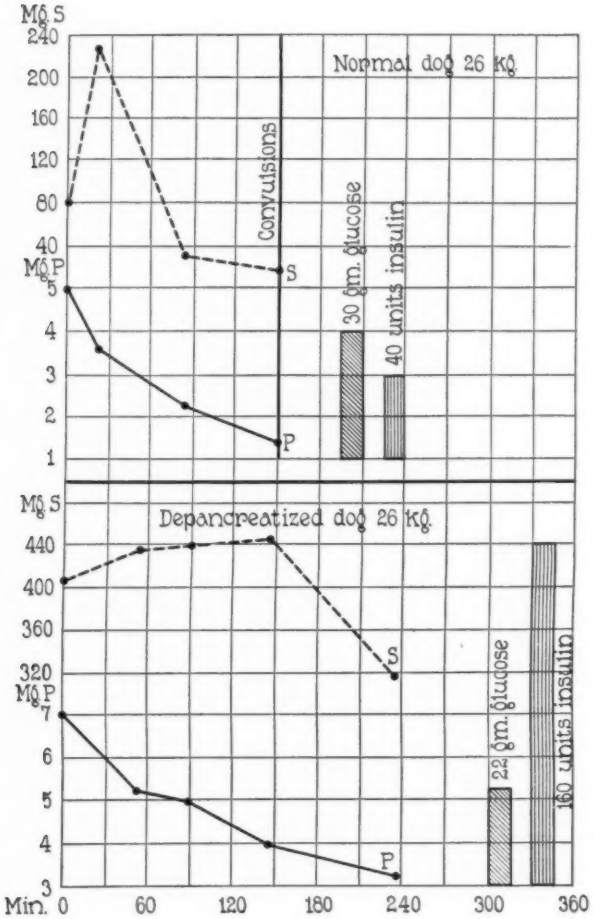
Mg S
240
200
160
120
80
40
Mg P
5
4
3
2
1
Normal dog 26 Kg.
Convulsions
S
P
30 gm. glucose
40 units insulin
Mg S
440
400
360
320
Mg P
7
6
5
4
3
Depancreatized dog 26 Kg.
S
P
22 gm. glucose
160 units insulin
Min. 0
60
120
180
240
300
360

CHART 10.

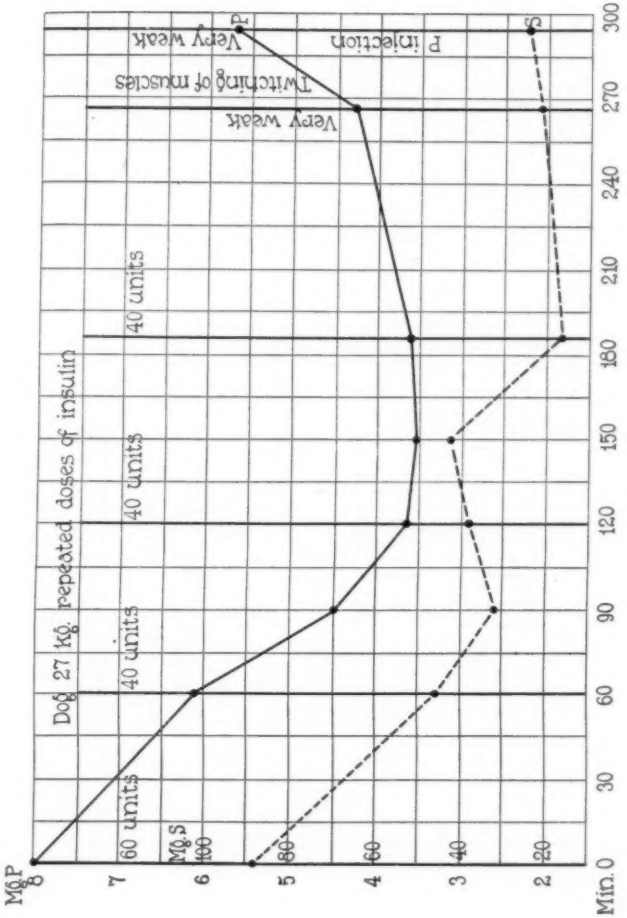
Dog 27 kg. repeated doses of insulin
60 units
40 units
40 units
40 units
Very weak
Twitching of muscles
Very weak
P injection
P
S
Mg P
Mg S
Min. 0
30
60
90
120
150
180
210
240
270
300

Chart 11.

tion; that is, insulin and adrenalin are not antagonistic in phosphate metabolism.

*Administration of Pituitrin.*—Cushing observed several years ago that there was a certain antagonistic action between the posterior lobe of the pituitary body and the pancreas. The work of Burn (11), referred to by Dale (12), and the work of Winter and

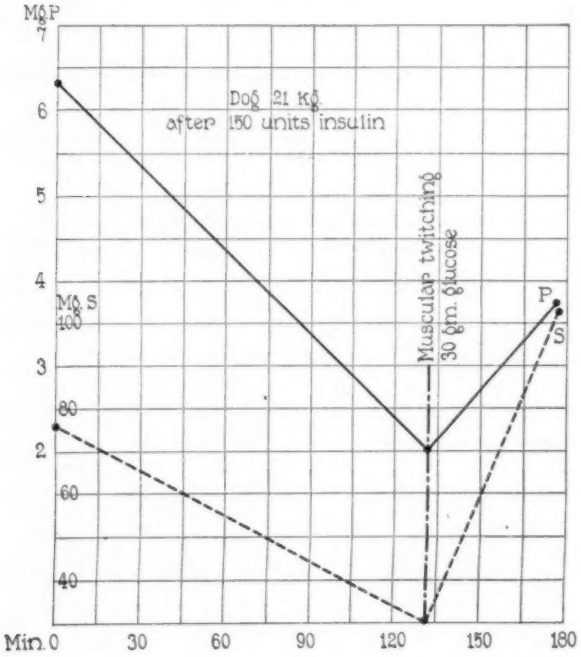


CHART 12.

Smith (7) showed definitely that there is an antagonistic action on carbohydrate metabolism between pituitrin and insulin. Study of the blood in normal dogs after the administration of Parke, Davis and Co. surgical pituitrin shows a mild hyperglycemia and a well marked increase in the inorganic blood phosphates (Chart 15). As regards phosphate metabolism pituitrin is therefore a direct antagonist of insulin. That the antago-

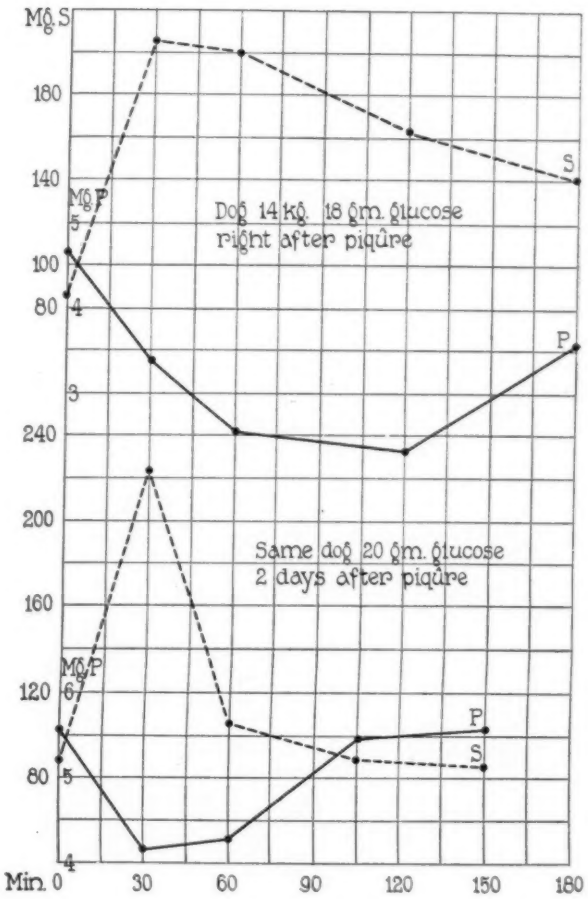
Mg. S
180
140
Mg. P
5
100
80
4
3
240
200
160
Mg. P
120
6
7
80
5
4
Min. 0
30
60
90
120
150
180
S
P
Dog 14 Kg. 18 gm. glucose
right after piqûre
P
Same dog 20 gm. glucose
2 days after piqûre
P
S

Chart 13.

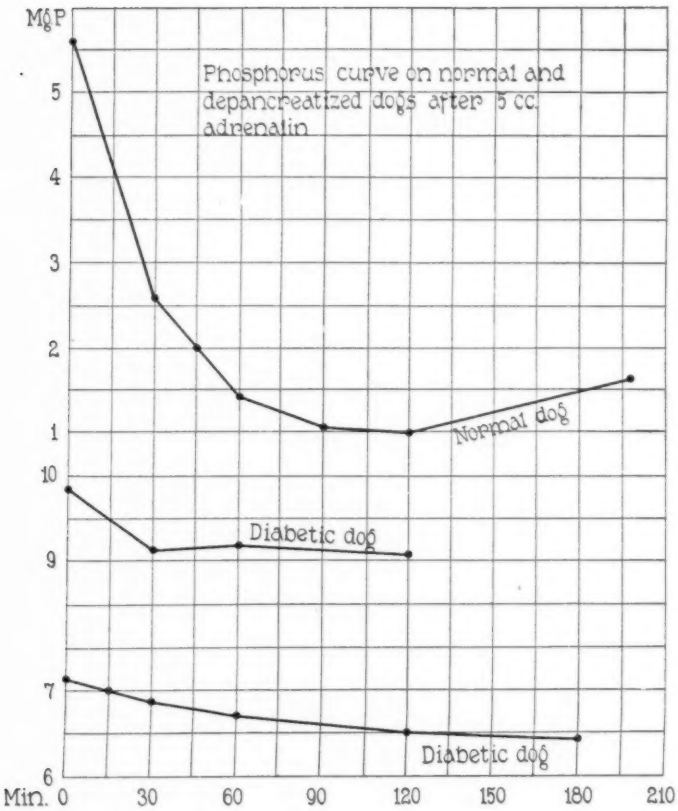
MgP
Phosphorus curve on normal and depancreatized dogs after 5 cc. adrenalin
5
4
3
2
1
10
9
7
6
Normal dog
Diabetic dog
Diabetic dog
Min. 0
30
60
90
120
150
180
210

CHART 14.

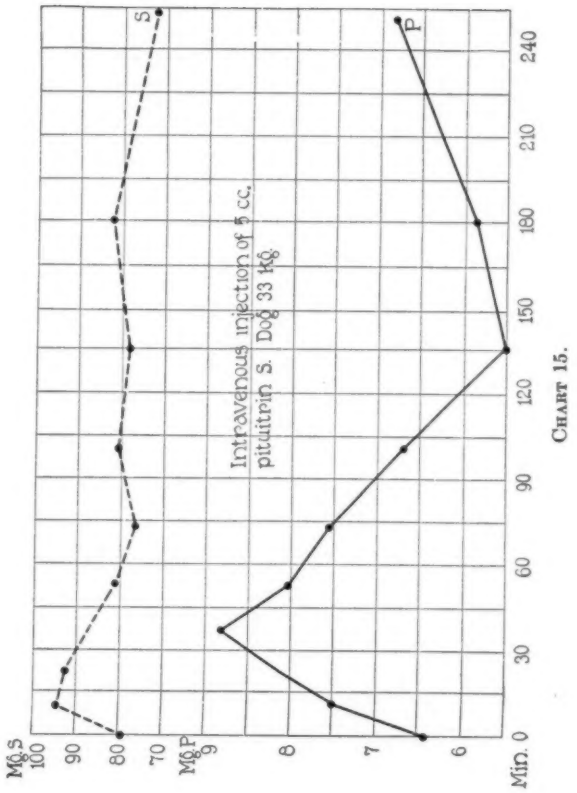
Intravenous injection of 5 cc. pituitrin S. Dog 33 Kg.
S
P
Mg.S
100
90
80
70
Mg.P
9
8
7
6
Min. 0
30
60
90
120
150
180
210
240

CHART 15.

nistic action is an active one and not a mere cessation of the demand for phosphates is shown by the large increase over the starting point. Even when pituitrin and insulin are injected together there is a temporary rise in the blood phosphates (Chart 16).

Elias and Weiss (1922) (13) reported that the intravenous injection of phosphate solutions in diabetics and alimentary hyperglycemias reduces the blood sugar and has a distinct beneficial effect upon the clinical picture. In attempting to confirm these observations phosphates and glucose were administered separately

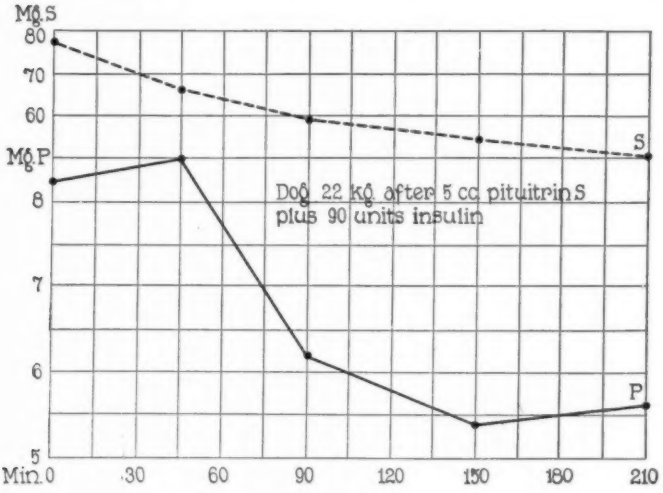


CHART 16.

and together in normal dogs. Glucose was better utilized in the presence of excess phosphates, and phosphates were better utilized in the presence of excess of glucose (Chart 17). On the other hand, phosphates administered to the depancreatized dog with a fasting sugar of 420 mg. show a blood phosphate curve which is identical with that seen in the normal dog. As far as the utilization of blood sugar after the administration of phosphates in the depancreatized animal is concerned, no such tendency was observed (Chart 18).

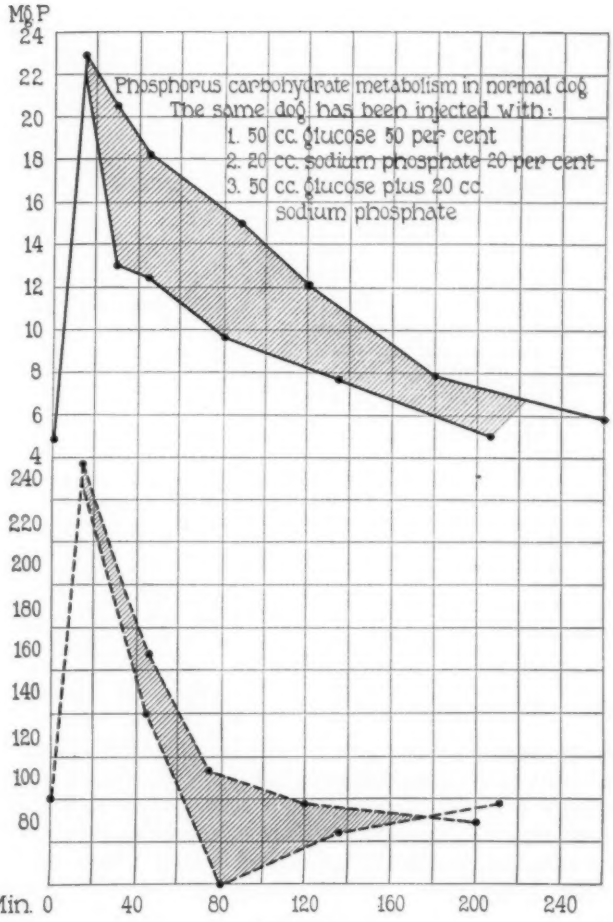
Mg P
24
22
20
18
16
14
12
10
8
6
4
240
220
200
180
160
140
120
100
80
Min. 0
40
80
120
160
200
240
Phosphorus carbohydrate metabolism in normal dog
The same dog has been injected with:
1. 50 cc. glucose 50 per cent
2. 20 cc. sodium phosphate 20 per cent
3. 50 cc. glucose plus 20 cc. sodium phosphate

Chart 17.

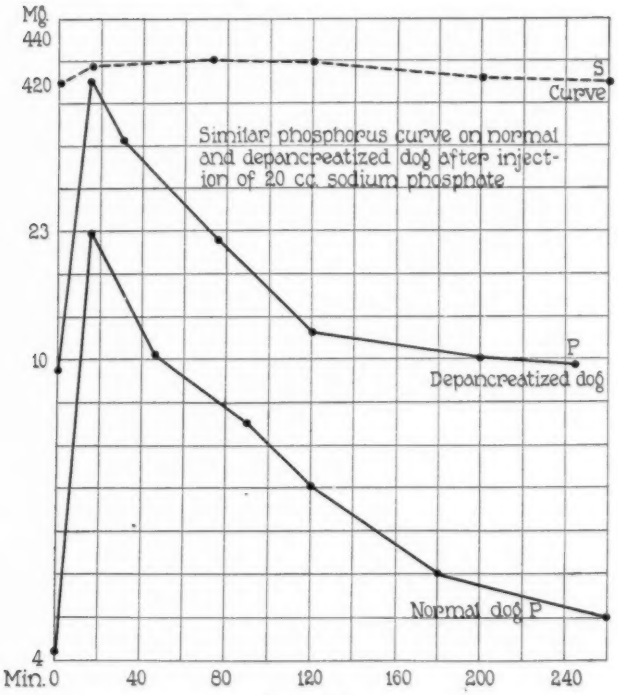


CHART 18.

DISCUSSION AND SUMMARY.

In the partially depancreatized dog and the diabetic patient of milder type the curve of inorganic phosphates is depressed more gradually and the depression lasts longer than in the normal individual. This variation in the blood phosphate curve is often more apparent than the variation from normal in the corresponding blood sugar curve. The prolonged depression in the phosphate curve in these cases resembles closely that following medullary puncture with its associated hyperglycemia so that it is probably explained by the fact that there is poor storage of glycogen in the liver with overstimulation of the pancreas. The more severe the diabetic state the greater is the effect upon the carbohydrate-

phosphate metabolism, and the level of blood phosphates approaches a straight line drawn parallel with the base line.

Decreased excretion of inorganic phosphates in the urine and the depression of inorganic blood phosphates during carbohydrate metabolism in normal animals and normal and diabetic individuals has been demonstrated by other investigators cited above. In following the blood phosphates in completely depancreatized dogs there is no appreciable fall unless insulin is administered. The demand for inorganic phosphates is apparent only when pancreatic hormone is available and carbohydrates are being utilized. It is unlikely that this utilization takes place in the liver since it was observed to take place after medullary puncture and in animals whose livers had been reduced to a functionless mass of fat.

Pituitrin as contrasted with adrenalin produces a large increase in the inorganic phosphate content of the blood so that, as regards phosphate metabolism, pituitrin is a direct antagonist of insulin.

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# THE ANTISCORBUTIC PROPERTIES OF EGGS.*

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(Received for publication, February 27, 1925.)

The presence of certain vitamins in eggs has been demonstrated by numerous investigators (1–8). Their work has been confined to studies of the fat-soluble and water-soluble B vitamins. A careful survey of the literature failed to show data concerning the antiscorbutic value of eggs. Many of the later tables (9, 10) compiled by leading investigators report the vitamin C content of eggs as being uninvestigated or unknown. The purpose of this experiment was to test the potency of the egg, white and yolk separately, in relation to its prophylactic and therapeutic value in scurvy.

## EXPERIMENTAL.

Since the vitamin content of eggs has been shown to be dependent on the vitamin content of the feeds fed to hens (11), the eggs used were from hens in an experimental lot which had been fed on an antiscorbutic ration. The technique and diet for the guinea pigs were the same as employed in a previous investigation (12).

## RESULTS.

Egg whites fed to guinea pigs in quantities as large as 20 cc. daily delayed the symptoms of scurvy no longer than did the basal ration. Egg yolk proved equally ineffective when given at the rate of 15 cc. daily. The therapeutic action of egg portions was also negative. All of the guinea pigs were autopsied, and the presence of scurvy was confirmed.

In this experiment we have not been able to demonstrate the

* Published with the approval of the Director of the Agricultural Experiment Station, Purdue University, Lafayette.



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presence of the antiscorbutic substance in the eggs of hens. The absence of this vitamin does not necessarily justify the supposition made by Plimmer, Rosedale, and Raymond (13) that "The growth of the chick in the shell to the day of hatching is one of the best indications that C-vitamin is not needed by the growing bird," because the possibility of synthesis of this factor by the chick is not excluded. Our findings do not support the statement of McCollum (14) that "Egg yolk is at least comparatively rich . . . . in water soluble C."

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# CONCERNING THE ADEQUACY OF SYNTHETIC DIETS FOR THE GROWTH OF THE CHICK.*

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The earlier attempts at studying the nutritional requirements of chickens were uniformly unsuccessful. Such studies meant that the birds must be reared in confinement, and the first investigators failed to accomplish that satisfactorily with any ration tried. Apparently Osborne and Mendel (1) were the first to attain any real degree of success in this type of investigation. Their chicks suffered from leg weakness but made gains in weight, one weighing 1,278 and another 1,267 gm. at the age of 178 days. These authors attributed their success in large measure to the use of a considerable quantity of roughage.

It was taken for granted from the first that vitamin B is required by chicks, but there has been some question as to the indispensability of vitamin A. Emmett and Peacock (2), however, established beyond question that chicks do require vitamin A. They obtained practically normal growth on a synthetic ration, and if any of the chicks were abnormal in any respect the fact was not noted. There is some doubt in our minds as to whether their ration would have been equally successful if begun with baby chicks.

A considerable advance in avian nutrition was made when Hart, Halpin, and Steenbock (3) announced that leg weakness can be prevented by the administration of cod liver oil, and that its symptoms are in some respects characteristic of rickets.

Not long afterwards Hughes (4) in a popular article described

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observations which led him to believe that leg weakness of the chick is in reality rickets, and that it is prevented by exposure to direct sunlight. The observations of Hughes have been abundantly confirmed by Hart, Steenbock, Lepkovsky, and Halpin (5).

At present, then, it is generally agreed that in addition to a proper adjustment of the supply of protein, salts, and energy, chickens require three vitamins, A, B, and D (antirachitic), unless they are exposed to ultra-violet rays. In that case the antirachitic vitamin is dispensable.

## EXPERIMENTAL.

On the 1st or 2nd day after hatching our chicks are transferred to a brooder located in a substantial stone building. The building is heated with steam and the brooder itself is warmed with an electric hot-plate. When the chicks are 6 to 8 weeks old they are transferred to wire cages in the same room; these cages are 2 feet square, and give practically no opportunity for exercise. We believe our experience abundantly establishes the fact that good growth can be obtained in severely restricted quarters. In order to reduce opportunity for variation in experimental conditions, none of our birds, except in special cases, has been exposed to direct sunlight, and we have relied almost entirely on cod liver oil for the antirachitic vitamin. All of our chicks are pure-bred, single comb White Leghorns.

There is always some question as to the freedom of nutrients from undesirable contamination, so we are including a brief description of the more questionable products, also of the salt mixture.

*Casein.*—A commercial product obtained from Lister Bros., New York City. So far as could be determined by tests on rats it was practically free of vitamins A and B.

*Corn-Starch.*—Buffalo Brand.

*Cod Liver Oil.*—Squibb preparation.

*Salt Mixture.*

| | per cent |
|---|---|
| Ca lactate | 48.90 |
| $K_2HPO_4$ | 29.30 |
| NaCl | 12.80 |
| Na citrate | 3.23 |
| Fe " | 2.44 |
| $MgSO_4$ | 3.32 |
| NaI | 0.01 |

Our earlier feeding trials were largely preliminary in nature, so we will omit a detailed description. Our chief concern at the time was the rôle of vitamin A in avian nutrition, and we confirmed the statement of Emmett and Peacock that this vitamin is indispensable for the chick.

We also gave some attention to the relation between nutrition and development of the feathers. Without exception in our experience the feathering becomes ruffled when chicks receive synthetic diets, and remains so for some weeks, but eventually becomes normal if the birds survive until maturity. This ruffling seldom appears, however, if any quantity of natural foodstuffs is included in the ration.

The first experimental series to be described in detail was begun March 12, 1924. The primary object was to repeat some of our earlier work under more favorable circumstances, and to determine whether the mortality could be lowered to an insignificant rate on rations of the type we are using. In that respect we feel that we have had some success. Another object was to substitute natural foodstuffs for a portion of our synthetic diets to see if such substitutions permitted a higher nutritional state than could be attained otherwise. There were five chicks in each lot and the rations employed had the following composition.

| Ration No. | 167 | 166 | 169 |
|---|---|---|---|
| | *per cent* | *per cent* | |
| Casein | 20 | 20 | Ration 167 + 2 egg yolks daily. |
| Corn-starch | 52 | | |
| Polished rice | | 52 | |
| Crisco | 10 | 10 | |
| Cod liver oil | 5 | 5 | |
| Dried yeast | 6 | 6 | |
| Salts | 4 | 4 | |
| Cellulose | 3 | 3 | |

Another group, designated as Lot 167 A, also received Ration 167, but except on Sunday was exposed for 2 or 3 hours to sunlight every suitable day. For convenience the different groups are designated by the ration they received. The mortality in Lots 167, 166, and 169 was zero. Of the five chicks in Lot 167 A, two refused to eat and they died on the 4th day. At autopsy

there were only traces of food in the alimentary tract. Another group of chicks had been started under identical conditions on a chick feed mixture devised for practical use, and three died within the 1st week. Of the survivors, one developed leg weakness during the 3rd week. These two were then added on April 6 to Group 167 A. 10 days later the chick suffering from leg weakness had completely recovered, and there were no further losses. The object of exposing this group to sunlight was to make sure that growth on a ration containing cod liver oil could not be improved

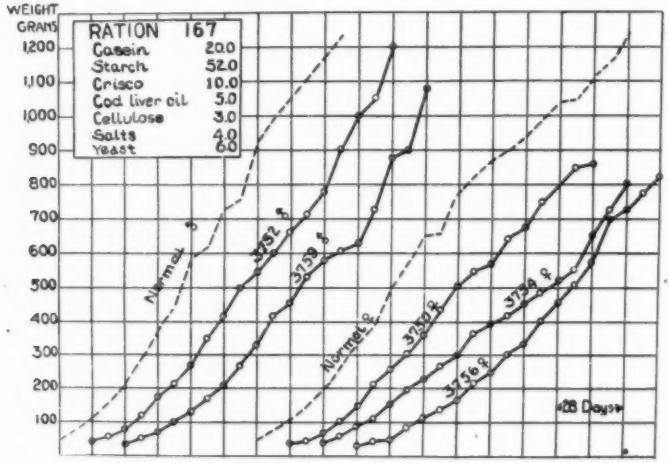


FIG. 1. Ration 167 is the basal diet in this series. The rate of growth was subnormal, and during the first 2 months the feathers were ruffled in the manner characteristic of chicks reared on synthetic diets. All were alert and healthy as long as they were under observation.

by exposure to sunshine. Conditions at this time were not favorable for our purpose. For some weeks the outdoor temperature was very low, and there were so many cloudy days that the chicks could be taken outside very little. However, this experiment was repeated later under favorable conditions, and with the same result. Those taken outside could not be distinguished in any way from those that received only indirect light. For some weeks, though the chicks were alert and apparently healthy, there was

pronounced ruffling of the feathers. A few gradually developed a characteristic deformity of the feet, crooked toes, and the rate of growth was subnormal.

Somewhat to our surprise Ration 166 proved the most successful in our experience. It will be noted that this mixture differed from Ration 167 in having polished rice substituted for cornstarch. All five chicks survived in perfect health, the average rate of growth is as rapid as is indicated on the standard growth

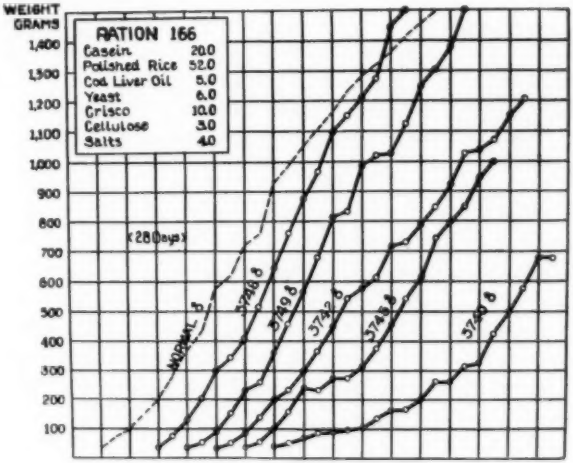


FIG. 2. It will be noted that the starch of the basal ration of No. 167 has been replaced with polished rice. This chart, therefore, should be compared with Fig. 1. As a result of the substitution of polished rice, four of the five chicks grew at practically the normal rate. All were feathered smoothly, and the specimens seemed entirely normal.

curves published, and the feathering was entirely normal at all times. The pigmentation of the skin was light, undoubtedly due as Palmer and Kempster (6) have shown to the lack of pigment in the diet. With this exception, however, the chicks appeared normal in every respect. One of the five was always decidedly undersized, but was alert and seemed entirely normal otherwise. Needless to say the only difference in the treatment accorded Lots 167 and 166 was in the ration. The adequacy of their rations may be compared by referring to Figs. 1 and 2.

The outcome of our experience with the lot receiving egg yolk was in the main highly satisfactory. The chicks grew more uniformly (see Fig. 3) than those receiving the rice diet, but at approximately the same average rate. One point seems worthy of note, however, the feathering was imperfect, in fact, ruffled to about the same extent as was noted in the chicks receiving Ration 167. It will be recalled that Lot 169 received the same basal ration as Lot 167, but was given two egg yolks daily in addition.

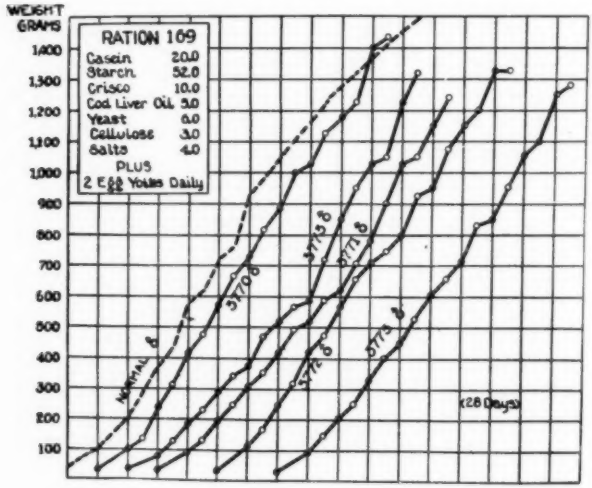


FIG. 3. When eggs were added to the basal diet growth proceeded at a normal rate. The feathering, however, was not entirely satisfactory.

We are uncertain how much importance should be attached to the condition of the feathers, though we have constantly borne it in mind. We are convinced, however, that the ruffled feathering we have noted is due to a nutritional inadequacy, and not to confinement, handling, or any other incidental factor.

The low mortality rate in the first 1924 series was unexpected, so we decided to repeat part of the work, but with some modifications. The most important of these was the incorporation of dried egg yolk in the ration, instead of feeding it separately.

The preceding series had been successfully reared on similar rations, containing 6 per cent yeast, so we were surprised when in a little less than 2 weeks a few of the chicks developed leg weakness. The symptoms indicated polyneuritis, but we were slow to believe the difficulty was due to a deficiency of vitamin B. During the preceding winter we had occasionally noted similar cases, but had failed to relieve them in any definite way by administering yeast. The symptoms were so suggestive of polyneuritis, however, that we again tried feeding additional yeast. Two chicks on the basal ration (No. 167) began in the 2nd week to show symptoms of leg weakness, and on the 13th day they were severely affected. No chick in that condition had ever recovered previously. A small quantity of dried yeast (not weighed) was made into a thin paste, and each affected chick was given 1 cc. of the yeast emulsion daily for 3 successive days. Both had then recovered.

As soon as it was reasonably certain that the additional yeast had improved the condition of these chicks, all rations in use at the time were changed by increasing the yeast to 8 per cent and decreasing the carbohydrate component correspondingly. This quantity of yeast was not sufficient, however, to afford complete protection against polyneuritis. We used 58 chicks in the second and third series together, and of these five died, though at least one did not die of polyneuritis. The other four did not exhibit the symptoms that are characteristic of that disease, but we believe the deaths were due either directly or indirectly to a lack of vitamin B. Another four developed more or less typical neuritic symptoms. One case was relatively mild and received no treatment, but the chick's legs became deformed and uncoordinated in a very characteristic way. The other three were on various occasions at the point of death, and displayed the symptoms that usually precede a fatal outcome, such as stupor and retraction of the head. They were successfully treated, however, either with yeast emulsion or a commercial preparation of a vitamin B concentrate, prepared according to the procedure of Osborne and Wakeman (7). From our view-point the surprising feature of these cases was the fact that growth may be only slightly interrupted, and still the neuritic symptoms may be quite marked. These symptoms were usually relieved promptly by the adminis-

tration of either dried yeast or the commercial preparation of vitamin B concentrate.

Since some of the chicks in the second series were plainly suffering from a lack of vitamin B it occurred to us that such a deficiency might account for the failure in our first series to secure perfect nutrition with synthetic diets. For that reason the feeding trials just described were repeated, again with slight modifications. The most important of these was the incorporation of a

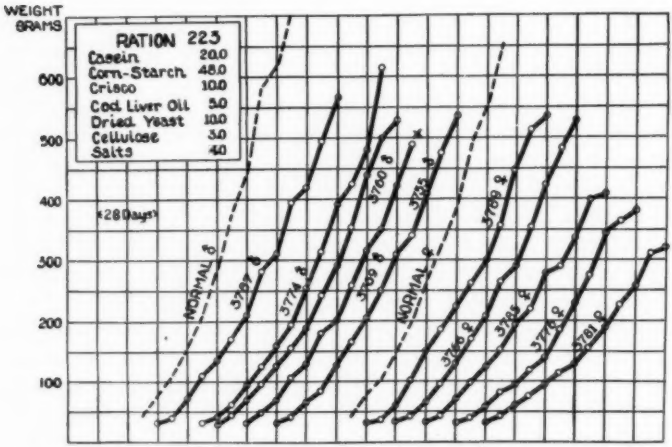


FIG. 4. Chicks 3787, 3789, 3785, 3776, and 3781 were each supplied daily with 40 mg. of the vitamin-Harris powder, in the form of the tablet preparation. This addition did not, however, have any perceptible effect. The yeast component was increased on the 16th day from 8 to 10 per cent of the ration, but we do not believe the change affected the growth rate.

slightly larger quantity (8 per cent) of dried yeast in the ration. 15 days after the experiment started, however, this was increased to 10 per cent. In addition to this, half of the chicks on the basal diet were given a sufficient quantity of the vitamin-Harris tablets each day to provide on the average 40 mg. of the vitamin concentrate. As shown in Fig. 4 the chicks receiving the additional vitamin B could not be distinguished in any way from the others. None of the chicks developed polyneuritis, but the rate of growth was still subnormal.

Another group received a ration very similar to the basal diet, No. 223, but with 5 per cent of the casein substituted by an equal weight of beef muscle that had been extracted repeatedly with ether, alcohol, and water. This was tried because it seemed possible that the more rapid rate of growth obtained when rice or egg yolk was incorporated in the diet may have been due to the protein factor. Beef muscle seemed favorable material with which to test that point, but the findings were entirely negative. In fact, two of the chicks developed leg weakness a few days after the yeast component had been increased to 10 per cent. As usual in such cases, however, the symptoms were relieved by administering additional vitamin concentrate or yeast emulsion.

The outcome of the trial with egg yolk was practically identical with the others, except that a case of leg weakness developed on the 50th day. A single feeding of 40 mg. of the vitamin B concentrate was sufficient to effect a temporary recovery, and no more was given at the time. 8 days later the chick was again in poor condition, and the feeding of yeast emulsion was begun. General recovery was immediate, and this specimen gained rather rapidly in weight, but it never regained control of its legs.

This and other similar cases we have observed have led us to believe that such injuries become permanent if not promptly relieved by a suitable curative agent. Yeast and yeast extracts have proven effective in our hands. If the supply of these materials is just below the border-line of safety over a long period of time we have concluded there is organic impairment and recovery cannot be brought about by any nutritional régime.

As to leg weakness itself, we are inclined to believe that though the nature of the injury may be specific, the symptoms are not, at least in the early stages. In our later work we have constantly fed cod liver oil, and have taken the precaution to mix it with the other constituents at intervals of not more than a week. Cases of leg weakness have not been infrequent, however, and in the early stages they are usually relieved by the administration of additional yeast. Knox and Lamb (8) have expressed similar views as regards the specificity of leg weakness.

It seems clear that the type of leg weakness we have described is entirely different from that induced by a lack of antirachitic vitamin. The acute phase is probably a symptom of polyneuri-

tis. Advanced cases, however, are not entirely relieved by the administration of vitamin B, though the health of the birds seems normal, and they may gain rapidly in weight. The legs, however, become characteristically deformed, and the chicks are almost helpless. Fig. 5 is included to make the description more complete.

FIG. 5. Chick 1959, age 71 days. Ration contained 10 per cent of dried yeast and 10 per cent of dried egg yolk. A condition of tonic spasticity is always characteristic of the leg muscles in the cases of polyneuritis we have observed. When the symptoms develop suddenly and the feeding of additional yeast or yeast extract is begun promptly, recovery is usually complete, or nearly so. If treatment is sufficiently delayed, however, we find that changes in the ration are ineffective so far as the paralysis is concerned, though the general well-being may be vastly improved.

## DISCUSSION.

We have successfully reared a considerable number of baby chicks on synthetic diets, and in our more recent attempts the mortality has been low. There is, however, considerable variability among individuals, and not infrequently we have observed unexpected nutritional disaster, usually identified as leg weakness.

The first 1924 series, it will be recalled, was reared on rations containing 6 per cent of dried yeast, and no case of leg weakness was observed. In later feeding trials we thought our failure to secure strictly concordant results was due to a variable content of vitamin B, in the yeast. Other evidence, obtained in parallel feeding investigations, points the same way, but does not indicate

any such difference as is indicated by the performance of our chicks. In our most recent feeding trials (not described in this paper), leg weakness developed on rations containing 15 per cent of yeast. It is impossible to believe that the yeast used in our first trials was 2.5 times as potent as our later supply.

We have also, in connection with a possible deficiency of vitamin B, given some consideration to the rate of growth obtained with our basal diets. It developed in our first 1924 series that the chicks on the basal ration (Fig. 1) did not grow at the normal rate. When egg yolk or polished rice was included in the diet, however, growth was markedly accelerated, and practically the optimum rate was attained. It seemed possible that our basal rations were deficient in vitamin B, so in the third series of the year, half of the chicks on the basal ration were supplied with an additional amount of this vitamin. None of the chickens in this group developed leg weakness, and the rate of growth did not seem to be affected in the least by the administration of the additional vitamin. We concluded, therefore, that this addition did not improve the ration in any way.

In our efforts to find wherein lay the deficiencies of our basal ration we have added certain natural foodstuffs to the diets, and in the two additions reported, obtained a normal rate of growth. As to the successful feeding of egg yolk, there is a multiplicity of possible explanations, and we will defer comment for the present. The more favorable outcome following the use of polished rice is unexpected from any point of view. This food contains starch; a low percentage of protein, though it is of good biological value; an insignificant quantity of mineral elements; and, from a quantitative standpoint, practically nothing else.

It is useless to speculate on the subject, but we are acting on the hypothesis that the nutritional requirements of the chick differ markedly in at least one respect from the known requirements of mammals. Evidence supporting such a supposition is no more than circumstantial, and we are acting on this theory partly because so far as we are aware there is no record of baby chicks attaining a normal growth rate when consuming synthetic diets. In this connection one naturally thinks of palatability, but from our point of view such an explanation only offers a name for the difficulty and affords no real explanation. A ration may be un-

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palatable merely because it is at least partially inadequate. To our minds the favorable outcome of feeding trials with ground rice substituted for the starch of synthetic diets supports this view.

As regards the cases of polyneuritis we have observed one feature was quite surprising to us. Although the symptoms may apparently develop quite rapidly, growth may nevertheless continue at a rate only slightly diminished.

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# THE METABOLISM OF IMIDAZOLES.

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## INTRODUCTION.

The fate of the imidazole ring in the body is an interesting question, since one of the most common amino acids—histidine—is an imidazole compound. One would think that it would be relatively simple to trace the metabolism of the imidazole ring because of its specific structure and its relative stability *in vitro;* but such has not been the case and, until recently, the work done upon this subject has been based largely on actual precipitation of the compounds in question—an inadequate method when dealing with small quantities—or upon rather crude colorimetric processes. The development of an accurate, yet fairly simple, colorimetric method of a high degree of sensitiveness for the estimation of imidazole compounds by Koessler and Hanke (1) has made possible the present attempt to add something to the knowledge of the metabolism of imidazoles. The fact that only a small portion of the imidazoles in the food is excreted in the urine of man suggested the idea that perhaps the kidneys would show a different permeability toward various simple imidazole derivatives that might be of some use as a possible basis for a renal function test. While such difference will be shown to exist, it does not seem practical for clinical use.

In the present study, histidine, methyl imidazole, imidazole lactic acid, and imidazole were estimated colorimetrically in the blood and urine of a dog, on a standard diet, after intravenous injection of these compounds. The renal permeability to histidine was almost nil even when the concentration of histidine in the blood was high, while a considerable amount of the other

compounds was excreted with a relatively low concentration in the blood.

Of these imidazole derivatives, histidine is the only one that is normally introduced into the animal organism. Its extreme value for the animal's normal existence can be deduced from the fact that hemoglobin contains nearly 10 per cent of this amino acid. The results to be recorded below suggest a disruption of the imidazole ring in the case of histidine. The stability of this ring, from a tissue standpoint, seems to depend upon the nature of the side chain; the shorter the side chain, the greater the stability.

## HISTORICAL.

Guggenheim (2) gives a general review of the literature on imidazoles. Engeland, in 1908 (3), isolated histidine from human urine and recognized the presence of other imidazoles of unknown constitution. Kowalevsky, in 1910 (4), Abderhalden and Einbeck, in 1909 (5), and Abderhalden, Einbeck, and Schmid, in 1910 (6), attempted to ascertain the fate of histidine when ingested or injected, but could only demonstrate an increase in urinary nitrogen. Very little of the histidine could be isolated from the urine. Dakin and Wakeman, in 1912 (7), showed an increased formation of acetoacetic acid by the isolated liver, when histidine was present in the perfusing fluid, and postulated a disruption of the imidazole ring. Urocanic, or imidazole acrylic, acid was discovered in the urine of a dog by Jaffe, in 1874 (8). Its origin from histidine was shown by Hunter in 1912 (9, 10), and it was definitely associated with the normal catabolism of histidine by Kotake and Konishi, in 1922 (11, 12). As regards imidazoles other than histidine, there is little in the literature except the work of Barger and Dakin, in 1916 (13), on imidazole carboxylic acid, and the pharmacological investigation of imidazole derivatives by Auvermann, in 1919 (14). The latter author studied imidazole itself and found that 25 per cent of injected imidazole (in the rabbit) was excreted in the urine. However, the colorimetric process used was quite crude, being sensitive only above 0.75 per cent imidazole.

## EXPERIMENTAL.

*Source of Imidazole Preparations.*—The histidine used was the dichloride obtained from the Special Chemicals Company of Highland Park, Ill. It was 100 per cent pure.

Methyl imidazole was prepared from zinc methyl imidazole hydroxide, the latter having been obtained from glucose, ammonia, and zinc hydroxide according to the directions given by Windaus and Knoop (15). The remainder of the process was the modification by Koessler and Hanke (1).

Imidazole lactic acid was prepared according to the method described by Fränkel (16).

Imidazole was prepared from crude glyoxal obtained by the action of nitric acid on paraldehyde, a slow oxidation requiring a week at room temperature, following the method of Behrend and Schmitz (17). The mixture was then treated with ammonia and formaldehyde for a week according to the method of Debus (18), yielding crude imidazole. After neutralizing with barium hydroxide and removing ammonia, formaldehyde, and water by distillation, the material was extracted in turn with alcohol, chloroform, and amyl alcohol, yielding finally crude imidazole hydrochloride. Purification was effected by converting this into the picrate, removing the picric acid by extraction with hot benzene and hydrochloric acid according to Koessler and Hanke (19) and preparing imidazole oxalate. The latter was recrystallized from hot water, yielding a white substance with a melting point of 225°C. A nitrogen determination by the Kjeldahl process on 0.1 gm. of the oxalate gave a nitrogen percentage of 17.46, the theoretical value being 17.72 per cent. The compound was therefore pure. From imidazole oxalate, imidazole hydrochloride was obtained by treating a hot solution of the former with calcium carbonate in excess and acidifying the filtrate with hydrochloric acid. This solution was evaporated down to a small volume so that 30 cc. contained 0.5 gm. of imidazole—the amount injected.

*Subject of Experiment.*—The entire series of experiments was conducted on the same female dog, which was in perfect health throughout the course of these experiments. She weighed 14.0 kilos at the beginning of this work, and 21.6 kilos 6 months later. The animal was kept on a standard diet throughout consisting of 500 gm. of ground beef, 500 gm. of bread mixed with 1 liter of the water in which the meat had been cooked, and extra water *ad libitum*, though little of the latter was drunk. All the urine was preserved under toluene. Catheterization was, of course, employed as far as possible, so that ordinarily there would be no urine voided in the cage. Addition of bone ash to the diet, 50 gm. daily, helped to keep the feces solid. There was never admixture of urine and feces. All injections of imidazoles were made intravenously into a saphenous vein and blood was drawn at intervals by syringe without exposing the vein. The animal was not fed until after the experiment was over, but the work was so arranged that the feeding took place at the same time every day, between 11.30 a.m. and 12 noon.

## *Method for Estimating Imidazoles in Urine and Blood.*

As regards the urine, the method used was identical in every respect with that described by Koessler and Hanke (20). A 100 cc. portion of the 24 hour urine was used, while the other specimens (2 hour urine) were always made up to 100 cc. volume before analysis. The colorimetric readings were usually made in duplicate or checked by using varying proportions of the final filtrate (always 100 cc.). The material was diluted so as to give a reading between 5 and 15 mm. when the unknown was set at 20 mm. in order to insure greater accuracy.

The imidazoles in the blood were determined by using 10 cc. samples of blood, immediately laked by mixing with 90 cc. of distilled water so that practically no clotting occurred. From then on the method was the same as in the case of the urine. Preliminary experiments showed that the lead acetate and sodium hydroxide precipitation method was more satisfactory, for removing protein and interfering substances, than heat and acetic acid, or trichloroacetic acid. With the two latter methods the colorimetric process would show a slight greenish color (in the absence of imidazoles) that was apparently due to a small amount of bile pigment left in the final filtrate, since extraction with amyl alcohol in acid solution resulted in the removal of all color-producing material. With the lead acetate and sodium hydroxide method, no imidazoles were found in 10 cc. samples of human and dog blood, while 100 per cent recovery could be made after the addition of 10 mg. of histidine dichloride to such quantities of blood.

*Normal Urinary Imidazole Output in the Dog.*

The urinary imidazole figures throughout this paper are, following Koessler and Hanke (20), calculated on the basis of a

TABLE I.

| 2 hr. urine. | | 22 hr. urine. | | 24 hr. urine. | |
|---|---|---|---|---|---|
| Volume. | Imidazole index. | Volume. | Imidazole index. | Volume. | Imidazole index. |
| *cc.* | | *cc.* | | *cc.* | |
| 34 | 4.3 | 573 | 59.0 | 607 | 63.3 |
| 68 | 6.7 | 510 | 71.2 | 578 | 77.9 |
| 41 | 6.7 | 624 | 71.8 | 665 | 78.5 |
| 62 | 5.4 | 597 | 53.7 | 659 | 59.1 |
| 38 | 4.0 | 650 | 64.7 | 688 | 68.7 |
| 52 | 7.6 | 695 | 68.1 | 747 | 75.7 |

hypothetical compound the color value of which is the mean color value of the four carboxylated imidazoles—histidine dichloride, imidazole acetic, -propionic, and -lactic acids. The reason for this is the fact that we do not know the identity of the major part of the urinary imidazole complexes. Therefore, the only fair thing to do is to take the average of the colorimetric readings given by the four imidazoles above and use it as an arbitrary standard color value for the urinary imidazole mixture. Thus, since 0.00001 gm. of histidine dichloride gives a reading of 5.0 mm. (Congo red-methyl orange standard); 0.00001 gm. of imidazole acetic acid, 11.2 mm.; 0.00001 gm. of imidazole

propionic acid, 13.6 mm.; and 0.00001 gm. of imidazole lactic acid, 9.3 mm.; the mean color value for 0.00001 gm. of these imidazoles is about 10.0 mm. Then, with the test cylinder set at 20 mm. each mm. of the standard comparison color is equivalent to 0.000001 gm. of the imidazole complex. The imidazole index, then, is the number of mg. of this complex in the 24 hour urine. The figures in the tables represent mg. of the hypothetical imidazole in the quantity of urine given in the column to the left of the imidazole value. The imidazole value or index was determined for a 2 hour period (9 to 11 a.m. as control for the period of experimentation), 22 hour period (11 to 9 a. m.), and the total 24 hour period, the sum of the first two. It can be seen from Table I that the normal variation is quite marked, yet within fairly definite limits.

*Effect of Injecting 1 Gm. of Histidine Dichloride.*

1 gm. of histidine dichloride in 15 cc. of neutral solution was injected intravenously. Table II shows the effect upon the blood and urine values. There was no noteworthy increase in urinary imidazoles above the maximum value of control periods.

*Effect of Injecting 2 Gm. of Histidine Dichloride.*

In this experiment, as seen from Table III, the blood values rose about 50 per cent higher than following the injection of 1 gm. Yet the urinary imidazole value showed little increase other than a slight rise in the 2 hour specimen. The urinary output was quite high in the control periods.

*Effect of Injecting 5 Gm. of Histidine Dichloride.*

35 cc. of a neutral solution containing 5 gm. of histidine dichloride were injected intravenously. There were no untoward symptoms. Although the concentration of histidine in the blood reached the high level of 45 mg. per 100 cc., the increase in urinary imidazoles could account for only about 150 mg. of histidine. See Table IV.

*Evidence that Histidine Actually Is Excreted as Such in the Urine after the Injection of a Large Amount.*—4.0 gm. of histidine dichloride, in 30 cc. neutralized solution, were injected intra-

TABLE II.

| Conditions. | Urine. | | | | | | Blood. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 hrs. | | 22 hrs. | | 24 hrs. | | Histidine in mg. per 100 cc. Normal = 0 Time after injection. | | | | |
| | Volume. | Imidazole index. | Volume. | Imidazole index. | Volume. | Imidazole index. | 5 min. | 15 min. | 30 min. | 60 min. | 120 min. |
| | cc. | | cc. | | cc. | | | | | | |
| Control. | 54 | 6.0 | 1,030 | 85.5 | 1,084 | 91.5 | | | | | |
| | 95 | 6.6 | 710 | 74.5 | 805 | 81.1 | | | | | |
| | 25 | 6.1 | 480 | 50.5 | 505 | 56.6 | | | | | |
| Injected 1 gm. histidine dichloride. | 22 | 9.1 | 875 | 84.9 | 897 | 94.0 | 16.0 | 13.0 | 10.0 | 8.0 | 11.0 |
| Control. | 39 | 6.8 | 525 | 49.9 | 564 | 56.7 | | | | | |
| | 30 | 4.4 | 655 | 60.9 | 685 | 65.3 | | | | | |

TABLE III.

| Conditions. | Urine. | | | | | | Blood. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 hrs. | | 22 hrs. | | 24 hrs. | | Histidine in mg. per 100 cc. Normal = 0 Time after injection. | | | | |
| | Volume. | Imidazole index. | Volume. | Imidazole index. | Volume. | Imidazole index. | 5 min. | 15 min. | 30 min. | 60 min. | 120 min. |
| | cc. | | cc. | | cc. | | | | | | |
| Control. | 52 | 6.3 | 1,255 | 92.9 | 1,307 | 99.2 | | | | | |
| | 99 | 8.2 | 915 | 84.2 | 1,014 | 92.4 | | | | | |
| Injected 2 gm. histidine dichloride. | 78 | 15.0 | 820 | 78.7 | 898 | 93.7 | 26.0 | 16.0 | 11.0 | 10.0 | 10.0 |
| Control. | 95 | 4.2 | 673 | 39.7 | 768 | 43.9 | | | | | |
| | | | | | 1,520 | 106.4 | | | | | |

TABLE IV.

| Conditions. | Urine. | | | | | | Blood. | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 hrs. | | 22 hrs. | | 24 hrs. | | Histidine in mg. per 100 cc. Normal = 0 Time after injection. | | | | |
| | Volume. | Imidazole index. | Volume. | Imidazole index. | Volume. | Imidazole index. | 5 min. | 15 min. | 30 min. | 60 min. | 120 min. |
| | cc. | | cc. | | cc. | | | | | | |
| Control. | 64 | 4.1 | 840 | 63.8 | 904 | 67.9 | | | | | |
| | 129 | 5.2 | 1,075 | 72.0 | 1,204 | 77.2 | | | | | |
| Injected 5 gm. histidine dichloride. | 50 | 88.0 | 870 | 78.3 | 920 | 166.3 | 45.0 | 36.0 | 27.0 | 19.0 | 15.0 |
| Control. | 51 | 5.5 | 950 | 53.2 | 1,001 | 58.7 | | | | | |
| | 45 | 3.9 | 905 | 54.3 | 950 | 58.2 | | | | | |

TABLE V.

| Conditions. | Urine. | | | | | | Blood. | |
|---|---|---|---|---|---|---|---|---|
| | 2 hrs. | | 22 hrs. | | 24 hrs. | | Histidine in mg. per 100 cc. Time after injection. | |
| | Volume. | Imidazole index. | Volume. | Imidazole index. | Volume. | Imidazole index. | 15 min. | 120 min. |
| | cc. | | cc. | | cc. | | | |
| Control. | 66 | Lost. | 870 | 62.6 | 936 | 62.6+ | | |
| | 71 | 7.2 | 725 | 85.5 | 796 | 92.7 | | |
| Injected 4 gm. histidine dichloride. | 71 | 26.6 | 840 | 104.2 | 911 | 130.8 | 21.0 | 10.0 |
| Control lost. | | | | | | | | |

venously. As seen from Table V, the 2 hour urine had an imidazole index of 26.6. After treatment of 5 cc. of final filtrate (acidified with a drop or two of concentrated HCl) with 1 cc. of

5 per cent sodium nitrite for 5 minutes at room temperature and 40 minutes in the water bath, then making up to standard volume and determining colorimetrically, it was found that the color had become intensified, an aliquot portion giving a reading of 11.3 mm. instead of 10.2 mm. before treatment, an increase of 11 per cent. Pure histidine dichloride, under these conditions, gives an increase of 24 per cent, due to oxidation of the $\alpha$-amino group to a hydroxyl group. Therefore, about half of the imidazole color in the 2 hour urine was due to histidine itself, though the actual quantity represented is not over 25 mg., a very small fraction of the quantity injected. Normal control urine gives no intensification by this method.

*Evidence that the Blood Imidazole, after Injection of Histidine, Is Histidine.*—4.0 gm. of histidine dichloride in 30 cc. volume were injected intravenously. The blood drawn within 5 minutes after injection contained 35.0 mg. per 100 cc. (estimated as histidine). Using the intensification process described above, an aliquot portion of the final filtrate gave a reading of 10.8 mm. after treatment with nitrous acid, instead of 8.8 mm. before such treatment—an increase of 23 per cent, the theoretical increase being 24 per cent for pure histidine. The blood drawn 2 hours after injection contained 9.0 mg. per 100 cc. and in this case before treatment an aliquot portion gave a reading of 2.2 mm. and after treatment 2.7 mm., with an intensification of 23 per cent. Therefore, all of the imidazole in the blood after injection of histidine is histidine itself.

The above series of experiments with histidine dichloride is representative of the entire number performed. It is, therefore, not necessary to detail the rest. In general it may be said that intravenous injection of histidine dichloride causes a sharp rise in blood histidine (normally 0) with a rapid fall so that at the end of 2 hours only a few mg. are present per 100 cc. Yet practically none of the histidine is excreted until 4 or 5 gm. are injected. When the concentration of histidine in the blood is sufficiently high, some of the amino acid is excreted. The kidney threshold value for histidine is unusually high. Even under the most favorable conditions only 3 per cent of the amino acid injected is excreted as such. The imidazole ring of histidine must either be rapidly disrupted or stored in the tissues and destroyed gradually.

Apparently no imidazole derivative—a catabolite of histidine—is eliminated in the urine with the imidazole ring intact, or an increased color would be obtained much above the normal values.

*Effect of Injecting Methyl Imidazole.*

1 gm. of methyl imidazole was neutralized with normal HCl and injected intravenously in a volume of 20 cc. Within a few minutes, marked dyspnea, salivation, nausea, vomiting of large quantities of bile-stained mucus, and generalized coarse tremors set in. The animal was weak and apathetic for several

TABLE VI.

| Conditions. | Urine. | | | | | | Blood. | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 hrs. | | 22 hrs. | | 24 hrs. | | Methyl imidazole in mg. per 100 cc. Time after injection. | | | |
| | Volume. | Imidazole index. | Volume. | Imidazole index. | Volume. | Imidazole index. | 5 min. | 30 min. | 60 min. | 120 min. |
| | cc. | | cc. | | cc. | | | | | |
| Control. | 29 | 3.1 | 480 | 62.4 | 509 | 65.5 | | | | |
| | 31 | 3.2 | 550 | 37.9 | 581 | 41.1 | | | | |
| Injected 1 gm. methyl imidazole. | 27 | 250 | 340 | 510 | 367 | 760 | 4.4 | 3.3 | 2.8 | 2.4 |
| Control. | 25 | 5.1 | 560 | 44.2 | 585 | 49.3 | | | | |
| | 27 | 3.0 | 310 | 34.7 | 337 | 39.7 | | | | |

hours and would not eat. The dog recovered completely 12 hours after the methyl imidazole was injected. In striking contrast to histidine, the urine for the 2 hour period after injection of methyl imidazole had an imidazole value of 250 mg., corresponding to about 0.1 gm. of methyl imidazole. The color was characteristic for methyl imidazole. Within 24 hours after injection, a total of about 0.304 gm. of methyl imidazole had been excreted, 30 per cent of the amount injected, indicating a low renal threshold or deficient capacity of the tissues to absorb or destroy this compound, in contrast to histidine.

XUM

*Proof that Methyl Imidazole Is Excreted as Such in the Urine.*—In the experiment described above (Table VI) it was estimated from the colorimetric determinations that 30 per cent of the methyl imidazole had been excreted in the urine in 24 hours. Of this, 0.204 gm. was present in the 340 cc.—the 22 hour specimen. A 50 cc. portion of this urine was subjected to a steam distillation, as described by Koessler and Hanke (1). 800 cc. were distilled over and, of this, 0.1 cc. gave a reading of 10.5 mm. (Congo red standard), corresponding to 0.212 gm. of methyl imidazole for the 340 cc. This value compares well with the 0.204 gm. obtained above by direct determination. Since the normal urinary imidazoles are not volatile with steam under these conditions, this is absolute proof that this was the substance excreted in the urine and responsible for the high color values.

*Effect of Injecting Imidazole Lactic Acid.*

1.0 gm. of imidazole lactic acid, neutralized and made up to 22 cc., was injected intravenously. No untoward symptoms were noted. The blood values were intermediate between those of methyl imidazole and histidine in injections of similar amounts. The urine taken 2 hours after injection showed 0.368 gm. of imidazole lactic acid as estimated from the color values, the color being characteristic for the compound in question. Within the next 22 hours practically no imidazole lactic acid was excreted. Apparently, then, much of the compound—37 per cent—had been excreted when the blood content was at its height. In 24 hours about 40 per cent had been excreted. See Table VII.

*Evidence that Imidazole Lactic Acid Is Excreted as Such after Injection.*—The final filtrate from the 2 hour specimen, containing 0.368 gm. of imidazole lactic acid, was subjected to a silver precipitation. The residue did not crystallize. This was then treated with the calculated amount of picrolonic acid (2 per cent alcoholic solution). A definite yellow crystalline compound precipitated on cooling. This was recrystallized from hot 50 per cent alcohol. The crystals melted, with decomposition, from 213–215°, while a control picrolonate made from the original imidazole lactic acid preparation melted, with decomposition, from 211–213°.

*Imidazole Is Volatile with Steam.*

Since methyl imidazole is volatile with steam, imidazole should also be volatile. Experiment has shown this to be the case. The method and apparatus were identical with that described by Koessler and Hanke (1). 10 mg. of imidazole oxalate were mixed with 5 gm. of $Na_2CO_3$ and 10 cc. of water. The mixture was subjected to a continuous steam distillation, the distillate being collected in 100 cc. portions. The first 100 cc. contained 5 mg. and the first 600 cc. contained 9.6 mg., 96 per cent of the amount originally introduced. This affords a method for the selective

TABLE VII.

| Conditions. | Urine. | | | | | | Blood. | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2 hrs. | | 22 hrs. | | 24 hrs. | | Imidazole lactic acid in mg. per 100 cc. Time after injection. | | |
| | Volume. | Imidazole index. | Volume. | Imidazole index. | Volume. | Imidazole index. | 5 min. | 30 min. | 120 min. |
| | cc. | | cc. | | cc. | | | | |
| Control. | | | | | 480 | 55.7 | | | |
| | | | | | 380 | 37.2 | | | |
| Injected 1 gm. imidazole lactic acid. | 40 | 340 | 440 | 81.8 | 480 | 421.8 | 11.0 | 7.5 | 5.8 |
| Control. | 19 | 4.8 | 940 | 59.2 | 959 | 64.0 | | | |
| | | | | | 490 | 28.4 | | | |

determination of imidazole that is, in every respect, just as characteristic as the one previously devised for methyl imidazole. It would, obviously, be impossible to determine, by this method, imidazole and methyl imidazole if they were present together in a mixture.

*Metabolism of Imidazole.*

Imidazole has not, heretofore, been estimated colorimetrically. It was necessary, therefore, to construct a table for this compound similar to those previously constructed for the other imidazoles

by Koessler and Hanke. The imidazole on hand was in the form of oxalate. The table was, therefore, constructed for the anhydrous oxalate from which the values for other salts can be easily calculated. The oxalic acid radical does not interfere with the colorimetric characteristics of the imidazole.

The color is characteristic. It is distinctly more yellow than that of any imidazole previously investigated. A standard indicator mixture containing 1.0 cc. of stock Congo red and 5.3 cc. of stock methyl orange in a total aqueous volume of 500 cc. was required to match the color produced by imidazole. The color develops slowly and is distinctly yellow at first. After 10 minutes,

TABLE VIII.

| Reading on colorimeter, test cylinder set at 20. | Imidazole oxalate. |
|---|---|
| *mm.* | *gm.* |
| 1.0 | 0.0000025 |
| 2.0 | 0.0000050 |
| 3.0 | 0.0000075 |
| 4.0 | 0.00001 |
| 5.0 | 0.0000125 |
| 6.0 | 0.000015 |
| 7.0 | 0.0000175 |
| 8.0 | 0.00002 |
| 9.0 | 0.0000225 |
| 10.0 | 0.0000250 |
| 11.0 | 0.0000275 |
| 12.0 | 0.00003 |

a color of maximum intensity is obtained which is stabile for about 5 minutes. Readings were taken during this interval. The readings are exactly proportional to the amount of imidazole present, but only over a rather limited range. Quantities of imidazole oxalate in excess of 0.00003 gm. give values that are slightly below the calculated value. In determining this substance, the dilutions must be so controlled that the readings are never in excess of 12 mm.

Table VIII gives the depth in mm. of the indicator solution and its equivalent in gm. of imidazole oxalate. The test cylinder is set at 20 mm. as in the case of the other imidazoles. The amount of imidazole can also be determined by means of the formula

$$I \times 0.0000025 = G$$

where $I$ is the depth of the indicator solution in mm. and $G$ is the number of gm. of imidazole oxalate in the test cylinder.

*Effect of Injecting Imidazole Hydrochloride.*

0.5 gm. of neutralized imidazole hydrochloride in a total volume of 35 cc. was injected intravenously. No untoward effects were observed. Diuresis occurred in the next 24 hours. Table IX shows the results. The blood imidazole reached a maximum of 3.3 mg. per 100 cc., and dropped to 2.4 mg. per 100 cc. at the

TABLE IX.

| Conditions. | Urine. | | | | | | Blood. | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 hrs. | | 22 hrs. | | 24 hrs. | | Imidazole in mg. per 100 cc. Time after injection. | | | |
| | Volume. | Imidazole index. | Volume. | Imidazole index. | Volume. | Imidazole index. | 5 min. | 15 min. | 30 min. | 120 min. |
| | cc. | | cc. | | cc. | | | | | |
| Control. | 82<br>89 | 3.0<br>3.5 | 750 | 54.7 | 832 | 57.7 | | | | |
| Injected 0.5 gm. imidazole. | 164 | 0.127 gm. imidazole. | 1,120 | 0.338 gm. imidazole. | 1,284 | 0.465 gm. imidazole. | 3.3 | 3.0 | 2.9 | 2.4 |

end of 2 hours. The 2 hour urine after injection gave a color value corresponding to 0.295 gm. of imidazole oxalate or 0.127 gm. of imidazole—about 26 per cent of the amount injected. In the next 22 hours the urine contained 0.338 gm. of imidazole, corresponding to 0.784 gm. of imidazole oxalate. In the 24 hours following injection, therefore, a total of 0.465 gm. of imidazole was excreted—93 per cent of the injected material. All these values were obtained by using the steam distillation process on aliquot portions of urine. The colors developed by the test portions of blood were absolutely typical of imidazole.

SUMMARY AND CONCLUSIONS.

Histidine, methyl imidazole, imidazole lactic acid, and imidazole itself were injected intravenously at different times into the same dog, which was on a standard diet. Of the four compounds used, methyl imidazole was distinctly toxic in a dosage of 50 mg. per kilo. Histidine was innocuous at more than five times this dose. The blood concentration of these imidazoles was highest after the injection of histidine and lowest in the case of the toxic methyl imidazole, when similar amounts were injected. Normally, no imidazoles can be demonstrated in the blood of dog or man. In each case the imidazole compound present in the blood was the one injected and none other. The blood contained imidazoles for only a few hours after injection. Of the compounds used, histidine was almost completely retained and metabolized even after the injection of large doses and in the presence of a high concentration in the blood. Methyl imidazole was excreted to the extent of 30 per cent in the first 24 hours after injection, imidazole lactic acid about 40 per cent, and imidazole itself 93 per cent. The highest relative excretion occurred in the first 2 hours after injection. In every case, the increased urinary imidazole output was due entirely to the presence of the same imidazole as the one injected. In other words, none of the compounds used is an intermediary in the metabolism of any of the others. The animal organism has a high capacity for destroying the imidazole ring, especially in histidine. It does not seem to possess the ability to destroy imidazole itself in the absence of a side chain.

To Dr. Karl K. Koessler I must express my gratitude for suggesting this problem and outlining its general features. I wish to thank Dr. Milton T. Hanke, without whose chemical assistance and advice the work would scarcely have been possible.

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XUM

# THE COMBINATION OF PHENOL RED AND PROTEINS.*

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## INTRODUCTION.

Despite the many applications of dyes to problems in the biological sciences, no quantitative work has been done to determine the nature and the factors influencing their combination. The original purpose of this investigation was to study the combination of phenolsulfonephthalein (phenol red) and the blood proteins. The elimination of this dye by the kidney has been extensively studied and used as a means of throwing light on the mechanism of renal function and, hence, a knowledge of its combinations with proteins seemed prerequisite for a complete exploitation of this mode of attack of the problem.

Moreover, with the exception of a few semiquantitative measurements (1), and the recent work of Lasnitzki and L. F. Loeb (2)—the latter based on surface tension measurements—no work had been done on adsorption on lyophile colloids. Experimental difficulties have prevented the quantitative study of these systems despite their great practical importance in vital phenomena. The effect of the hydrogen ion concentration, too, as we shall see later, plays a part quite different from that in lyophobic systems, which has been hitherto unrecognized.

### *Adsorption of Phenol Red in Macroheterogeneous Systems.*

As a preliminary to determining the adsorption of phenol red in hydrophilic systems, its adsorption from aqueous solution by blood

* A preliminary account of this work was presented before The Johns Hopkins Medical Research Society, March 20, 1925.



XUM

charcoal and undissolved casein and gelatin was determined. The experimental procedure was that usually employed.

To weighed amounts of the adsorbent in glass-stoppered bottles were added measured volumes of solution of known dye concentration and the mixture was mechanically shaken for 24 hours in a thermostat. After settling, the supernatant liquid was centrifuged and analyzed with a Duboscq colorimeter. In the case of gelatin, a known amount of dye in a buffer solution (pH 3.97) was added to weighed quantities of granular gelatin and allowed to stand in an ice chest at 7°C. for 2 days with occasional shaking. The solution was then separated from the swollen granules and analyzed colorimetrically. It was assumed that the solution was imbibed in a concentration equal to that of the separated liquid and the dye thus left unaccounted for was held by the gelatin (3).

In the case of all of these substances, combination of the dye takes place in accord with the usual adsorption isotherm: $\frac{x}{m} = Kc^{\frac{1}{n}}$. Throughout this paper, $x$ is expressed in millimols of dye adsorbed by $m$ gm. of adsorbent; $c$ is the equilibrium concentration in millimols per liter; and $K$ and $\frac{1}{n}$ are constants. The experimental results are given in Tables I to IV. In Fig. 1, $\log \frac{x}{m}$ is plotted against log c, giving the linear curves characteristic of behavior in accord with the above parabolic equation.

The two varieties of casein employed (C. P. Merck, *nach* Hammersten, and the ordinary technical variety) gave two different curves, each of which conforms with the above formula. This difference in behavior is quite analogous to that obtained by the use of various kinds of charcoal or other inert adsorbents of varying physical texture. As in the case of these substances, the amount adsorbed depends on the physical state of the adsorbent; crystallinity tending to decrease the adsorption power. The more amorphous casein, similarly, shows the greater adsorption. This variation in behavior is readily explainable, if we consider adsorption as a manifestation of certain residual forces which in amorphous substances tend to be neutralized by the adsorption of other substances. In crystalline bodies, on the other hand, these forces are spent in maintaining the structure of the *raumgetter* and are no longer free to hold other substances.

TABLE I.

*Adsorption of Phenol Red in Water on Blood Charcoal at 32.0°C.*

| $c$ | $\frac{x}{m}$ |
|---|---|
| 0.00223 | 0.0425 |
| 0.0113 | 0.0690 |
| 0.0455 | 0.100 |
| 0.0547 | 0.106 |
| 0.234 | 0.143 |

TABLE II.

*Adsorption of Phenol Red in Aqueous Solution on Casein (Technical) at 32°C.*

| $c$ | $\frac{x}{m}$ |
|---|---|
| 0.00606 | 0.00123 |
| 0.0138 | 0.00305 |
| 0.0353 | 0.00602 |
| 0.0766 | 0.0121 |
| 0.179 | 0.0235 |

TABLE III.

*Adsorption of Phenol Red in Aqueous Solution on Casein (Highest Purity Pfanstiehl) at 32.0°C.*

| $c$ | $\frac{x}{m}$ |
|---|---|
| 0.0107 | 0.00117 |
| 0.0265 | 0.00293 |
| 0.0559 | 0.00580 |
| 0.116 | 0.0115 |

TABLE IV.

*Adsorption of Phenol Red in Aqueous Buffered Solution (pH 3.97) on Granular Gelatin at 7°C.*

| $c$ | $\frac{x}{m}$ |
|---|---|
| 0.0138 | 0.000145 |
| 0.0250 | 0.000316 |
| 0.0612 | 0.000719 |
| 0.0809 | 0.00118 |
| 0.113 | 0.00159 |
| 0.311 | 0.00370 |
| 0.420 | 0.00433 |

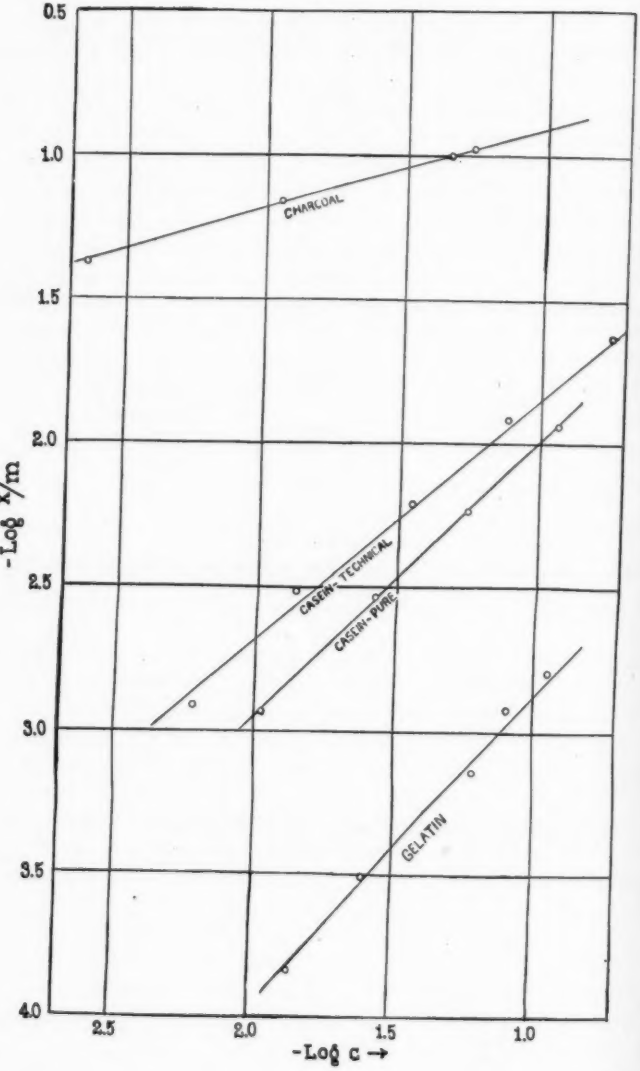
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
-Log x/m
2.5
2.0
1.5
1.0
-Log c →
CHARCOAL
CASEIN-TECHNICAL
CASEIN-PURE
GELATIN

FIG. 1.

Technical casein retains some of the acetic acid used in its preparation and hence the solution obtained by shaking water with it is more acid than that obtained by similarly treating the purified variety. In the samples used in this work this variation amounted to 0.5 pH. Hence, the technical variety would be expected to adsorb somewhat more of an acid dye. The variation obtained is, therefore, in part also due to this effect.

*Adsorption of Phenol Red by Lyophile Colloids.*

As has been pointed out by Freundlich (4) no direct measurement of adsorption by the micellæ of an hydrophile colloid, without simultaneous precipitation, has been demonstrated. That such adsorption does take place has, however, been indicated by surface tension measurements (5) and dialysis experiments (1, 6, 7). A qualitative optical proof of the adsorption of dyes on colloids in solution may be easily demonstrated in the case of phenol red by comparing its color in an aqueous solution with another solution of equal concentration to which has been added some protein. Adjustment for any optical effect due to the presence of the protein may be accomplished by arranging a tube containing the protein solution alone, behind the aqueous dye solution and a similar tube of water behind the protein-dye solution.

In order to study adsorption by lyophile colloids directly, one must (4) separate the intermicellar fluid from the micellæ and determine the concentration of the substance, whose adsorption is being studied, in this filtrate. Despite the apparent ease with which this might be carried out by means of ultrafiltration through one of the many available membranes impermeable to proteins, no practical success has been obtained by this method. This failure is due to the adsorption by the membranes, themselves, which usually takes place to such an extent as to preclude their use.

In the present investigation collodion membranes, prepared by the method of Marshall and Vickers (8), were found to be applicable to a study of the adsorption of phenol red, due to the comparatively slight extent to which it is adsorbed on the filter. To various protein solutions were added weighed amounts of dye

XUM

and the resulting solutions ultrafiltered under a pressure of 150 to 200 mm. of Hg. The solution in the filter was often renewed to prevent its concentration in respect to dissolved colloid. The filtration was continued until the ultrafiltrate was in equilibrium with the original solution as determined by the constancy of its concentration. Knowing the amount originally present in a given volume and the amount of protein, $m$, present in this volume, $\frac{x}{m}$ could be calculated from the determination of $c$, the concentration of dye in the ultrafiltrate.

The results for blood albumin and blood serum (pig) are given in Tables V and VI. The logarithmic curves are given in Fig. 2 in which $\log \frac{x}{m}$ is plotted against $\log c$. The results are seen to follow (within experimental error) the usual adsorption formula. The results with blood serum are obtained by adding varying amounts of dye to the pure serum. If one varies $m$, by diluting the serum, the results, if plotted on a logarithmic scale, no longer follow a linear relationship, though for any given dilution, this linear relationship holds for $c$ variable. This behavior is due in part, if not entirely, to changes in pH attendant on dilution of the serum which we shall see later greatly affects the adsorption.

The outstanding characteristic of the combination of phenol red and the various proteins studied, as compared with the results obtained with more inert substances like charcoal, wool, etc., is the rather high value of the constant $\frac{1}{n}$ in the adsorption isotherm. This is shown in the slopes of the curves of Figs. 1 and 2 which are seen to approach an angle of 45° very closely. From the formula

$$\log \frac{x}{m} = \log K + \frac{1}{n} \log c$$

it is obvious that when $\log c = 0$, $\log \frac{x}{m} = \log K$; *i.e.*, $\log K$ is the intercept on the $y$-axis and the slope of the curve representing this equation is, therefore, $\frac{1}{n}$. When the slope is 45°, $\frac{1}{n} = 1$ and disappears from the equation. The usual value of $\frac{1}{n}$ for charcoal

TABLE V.

*Adsorption of Phenol Red by a 3.4 Per Cent Blood Albumin (Difco) Solution at Room Temperature.*

pH 6.10.

| $c$ | $x$ |
|---|---|
| 0.0104 | 0.0588 |
| 0.0174 | 0.0890 |
| 0.0232 | 0.113 |
| 0.0314 | 0.127 |
| 0.0464 | 0.161 |
| 0.0983 | 0.219 |
| 0.109 | 0.257 |
| 0.145 | 0.277 |
| 0.201 | 0.324 |

TABLE VI.

*Adsorption of Phenol Red by Blood Serum (Pig) at Room Temperature.*

pH 7.85.

| $c$ | $x$ |
|---|---|
| 0.0080 | 0.0239 |
| 0.0178 | 0.0628 |
| 0.0346 | 0.127 |
| 0.0745 | 0.243 |
| 0.121 | 0.354 |

TABLE VII.

*Effect of pH on the Adsorption of Phenol Red by Charcoal. Original Concentration of Dye, 0.798 Millimol per Liter; Charcoal Used, 0.5 Gm.*

| pH | $c$ Equilibrium concentration. | $\frac{x}{m}$ adsorbed per gm. of charcoal. |
|---|---|---|
| | *millimols per l.* | *millimols* |
| 1.18 | 0.0564 | 0.148 |
| 2.18 | 0.0676 | 0.146 |
| 2.99 | 0.0878 | 0.142 |
| 5.24 | 0.129 | 0.134 |
| 6.40 | 0.180 | 0.124 |
| 7.02 | 0.297 | 0.100 |
| 10.24 | 0.670 | 0.0255 |
| 10.36 | 0.702 | 0.0191 |

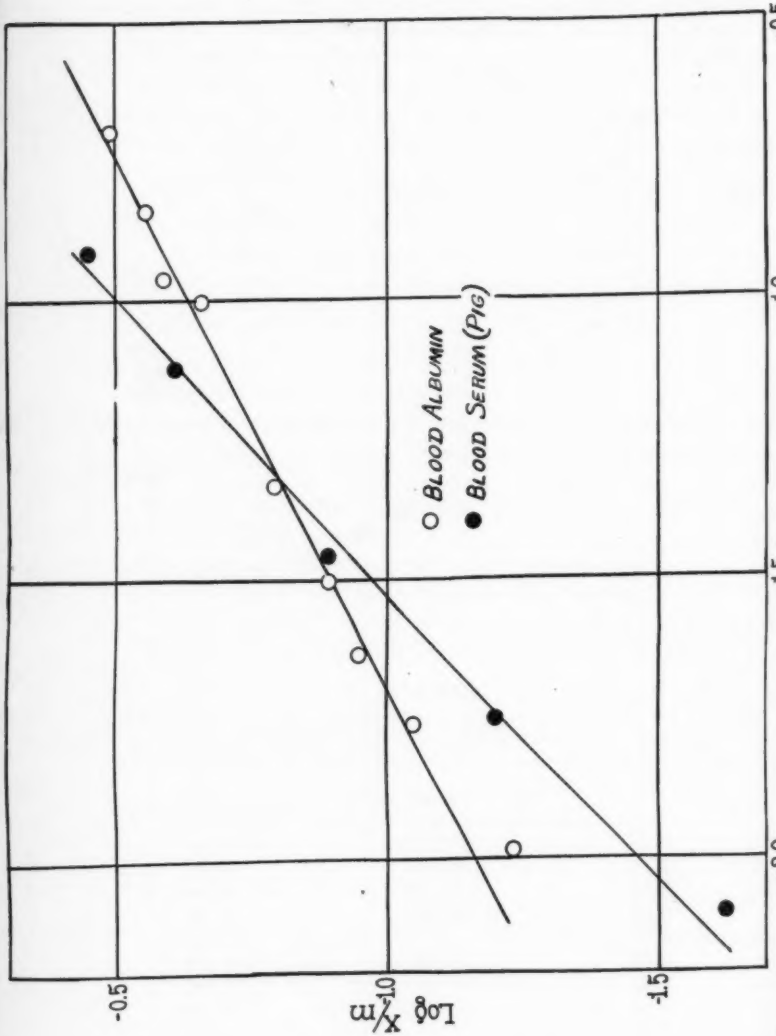


Fig. 2.

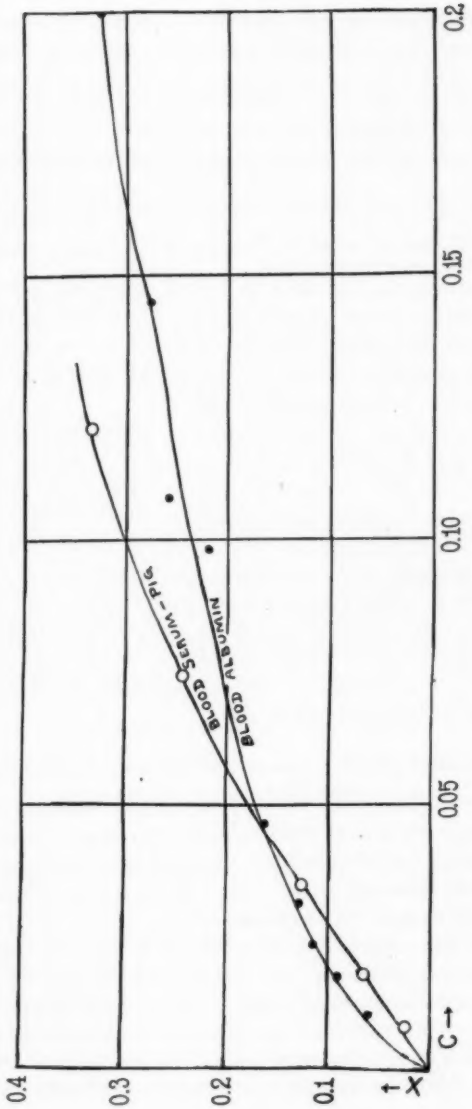


FIG. 3.

-0.5
-1.0
-1.5
Log c →
-2.0
FIG. 2.

and similarly inert substances is about 0.5 as seen in the curve for charcoal (Fig. 1). In the case of the adsorption of gases, however, $\frac{1}{n}$ approaches unity with increasing temperature as shown in the work of Titoff (9), Homfray (10), and others.

As a consequence of the close approach to unity of the value of $\frac{1}{n}$, we might as an approximation express adsorption on proteins by the simple formula $\frac{x}{m} = kc$. The approach to agreement with this approximation may be seen in Fig. 3 where the results of Tables V and VI for blood albumin and pig serum are plotted.

Similar curves have been obtained by Lasnitzki and Loeb (2) for adsorption of aliphatic acids by starch and gelatin as determined from surface tension measurements.

The constancy of the ratio between the concentration of the free dye and that combined with the protein, at constant pH, might suggest that one were dealing with a simple Donnan membrane equilibrium, which has been applied so successfully in interpreting the combination of proteins with acids and alkalies. However, on attempting to correlate quantitatively the results obtained in this investigation and those demanded on the basis of membrane equilibria, it becomes apparent that such interpretation is impossible and direct combination must be invoked as has been shown recently in the case of metallic salts by Northrop and Kunitz (11) and Pauli and Schön (12).

*Effect of the Hydrogen Ion Concentration on the Adsorption of Phenol Red by Charcoal and Proteins.*

The only work cited in the literature on the effect of hydrogen ion concentration on the adsorption of dyes by proteins are the meager qualitative experiments of Bethe (7) on the diffusion of acid and basic dyes in the presence of proteins. This worker found that diffusion of acid dyes through parchment was increased by the addition of alkali and diminished by acid; and *vice versa* with basic dyes. Negative adsorption occurred in strongly alkaline solution. This effect of acid and alkali gives continuous results with such inert adsorbents as charcoal which are themselves unaffected by the acid or alkali (13). It has been hitherto assumed

that the same result is obtained in the case of proteins. However, the effect of acid and alkali on the protein molecule may exert the predominating influence in solutions deviating greatly from neutrality.

To solutions of the various proteins, containing the same original concentration of dye, were added varying amounts of hydrochloric acid or sodium hydroxide. These solutions were ultrafiltered as explained above, and the amount of dye combined with the protein determined. The pH of the solution was also determined electrometrically. In the case of gelatin the procedure described above was followed.

Adsorption on charcoal was also studied as an inert comparison substance. To 0.5 gm. samples of blood charcoal were added 100 cc. of a dye solution containing 0.798 millimol of dye per liter brought to varying pH and the adsorption measured as described before. Due to destruction of phenol red in strongly alkaline solutions, results could not be obtained beyond the limits recorded in this investigation. The results with charcoal are given in Table VII. In Fig. 4 are plotted the millimols of dye adsorbed per gm. of charcoal against the pH of the solution.

It should be noted that curves such as Figs. 4 and 5 do not accurately define the change of adsorption power with varying pH, inasmuch as the variance in the equilibrium concentration, $c$, makes the values of $x$ incomparable. To define the relation between adsorptive power and pH more exactly, the values of $x$ found must be recalculated in terms of some arbitrarily fixed value of $c$ by means of the formula $\frac{x}{m} = kc^{\frac{1}{n}}$ (assuming $k$ and $\frac{1}{n}$ to be constant when pH varies). However, since the original concentrations of adsorbent and dye were always the same for the various pH values, the graphs given indicate the general change of adsorption sufficiently well for the purpose of this paper.

As seen in Fig. 4 the adsorption of phenol red on charcoal changes progressively with change in pH. This behavior can best be explained as follows. Let us consider adsorption of the dye to take place by an adsorption of the organic anion, either directly or by the displacement of hydroxyl ion as suggested by Bartell and Miller (14). The addition of alkali will affect adsorption in two ways. In the first place, increasing [OH] increases

the [OH] adsorbed, thus diminishing the concentration of the adsorbed organic cation; and secondly, by affecting the dissociation of the dye there will be a stronger electrostatic attraction between the cation (*e.g.*, $Na^+$, if NaOH were used) and the organic anion. Since the dissociation of the dye-salt is great in alkaline solution, this electrostatic charge between adsorbed

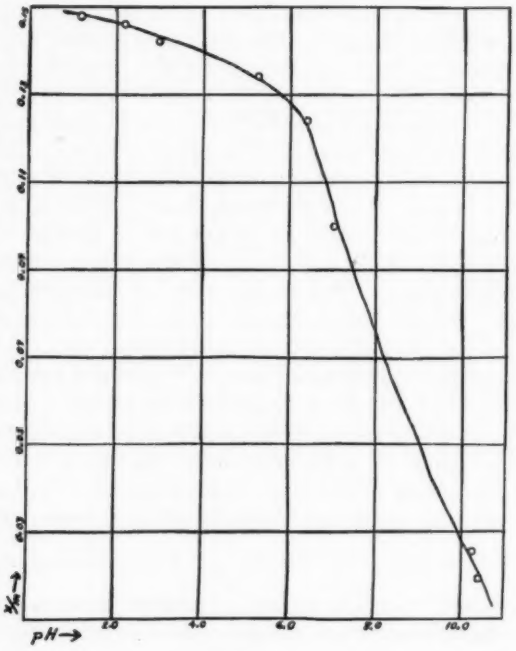


FIG. 4. Effect of pH on the adsorption of phenol red by charcoal.

anion and free cation will be very great and thus diminish the extent of the adsorption of the former. On adding acid, more of the undissociated dye-acid is formed and being but slightly dissociated, there will be but a slight tendency for the hydrogen ion to remove the anion from its union with the charcoal, and its adsorption will, therefore, be great. These facts, it will be seen,

TABLE VIII.

*Effect of pH on the Adsorption of Phenol Red by Gelatin (Undissolved Granules) at 7°C. Original Concentration of Dye, 0.1596 Millimol per Liter; Gelatin Used, 2 Gm.; Solution Added, 100 Cc.*

| pH | $c$ Equilibrium concentration. | $\frac{x}{m}$ adsorbed per gm. of gelatin. |
|---|---|---|
| | *millimols per l.* | *millimols* |
| 0.85 | 0.152 | 0.00038 |
| 1.25 | 0.125 | 0.00173 |
| 1.64 | 0.117 | 0.00213 |
| 2.46 | 0.0825 | 0.00386 |
| 3.40 | 0.0825 | 0.00386 |
| 3.77 | 0.0798 | 0.00399 |
| 4.94 | 0.120 | 0.00198 |
| 5.02 | 0.128 | 0.00158 |
| 5.32 | 0.138 | 0.00108 |
| 5.37 | 0.138 | 0.00108 |
| 8.90 | 0.186 | 0.00132 |

TABLE IX.

*Effect of pH on the Adsorption of Phenol Red by Blood Albumin (Difco). Original Concentration of Dye, 0.1596 Millimol per Liter; Albumin Concentration, 36.4 Gm. (Dry Weight) per Liter.*

| pH | $c$ Equilibrium concentration. | $\frac{x}{m}$ adsorbed per gm. of albumin. |
|---|---|---|
| | *millimols per l.* | *millimols* |
| 1.10 | 0.0452 | 0.00315 |
| 1.35 | 0.0285 | 0.00361 |
| 2.15 | 0.0194 | 0.00386 |
| 3.26 | 0.0112 | 0.00408 |
| 3.78 | 0.0101 | 0.00411 |
| 4.12 | 0.0152 | 0.00397 |
| 4.14 | 0.0138 | 0.00401 |
| 4.41 | 0.0213 | 0.00380 |
| 5.14 | 0.0290 | 0.00359 |
| 5.33 | 0.0330 | 0.00348 |
| 6.10 | 0.0378 | 0.00335 |
| 6.98 | 0.0412 | 0.00325 |
| 7.43 | 0.0428 | 0.00321 |
| 7.88 | 0.0511 | 0.00298 |
| 9.40 | 0.124 | 0.000979 |

are in accord with the findings as shown in Fig. 4. The same method of reasoning, *mutatis mutandis*, may be applied to the case of basic dyes.

In the case of proteins one would expect the same relations to hold. However, since the adsorbent itself is changed by a change

TABLE X.

*Effect of pH on the Adsorption of Phenol Red by Egg Albumin (Soluble Merck). Original Concentration of Dye 0.1596 Millimol per Liter; Albumin Concentration, 20 Gm. per Liter.*

| pH | $c$ Equilibrium concentration. | $\frac{x}{m}$ adsorbed per 1 gm. of albumin. |
|---|---|---|
| | *millimols per l.* | *millimols* |
| 1.25 | 0.0527 | 0.00535 |
| 1.48 | 0.0346 | 0.00625 |
| 2.13 | 0.0293 | 0.00652 |
| 2.35 | 0.0346 | 0.00625 |
| 3.03 | 0.0399 | 0.00599 |
| 3.73 | 0.0505 | 0.00546 |
| 4.30 | 0.0771 | 0.00413 |
| 5.13 | 0.0957 | 0.00320 |
| 5.49 | 0.125 | 0.00173 |
| 6.19 | 0.144 | 0.00078 |

TABLE XI.

*Effect of pH on Adsorption of Phenol Red by Blood Serum (Pig). Original Concentration of Dye, 0.1596 Millimol per Liter.*

| pH | $c$ Equilibrium concentration. | $x$ adsorbed per 1 liter of serum. |
|---|---|---|
| | *millimols per l.* | *millimols* |
| 6.81 | 0.0242 | 0.135 |
| 7.41 | 0.0287 | 0.131 |
| 7.46 | 0.0287 | 0.131 |
| 7.85 | 0.0309 | 0.129 |
| 8.07 | 0.0348 | 0.125 |
| 10.06 | 0.0997 | 0.060 |

in pH, the final adsorption obtained will be the resultant of these two effects. *A priori* we can consider adsorption to be a maximum at the isoelectric point and to decrease with addition of acid or alkali due to the ionic condition which the protein then assumes.

If the decrease in adsorption is very pronounced, as is to be expected, its effect should predominate in causing a diminution in adsorption with increasing acidity beyond the isoelectric point. The maximum adsorption will occur at or on the acid side of the isoelectric point since on this side, increased acidity, by its effect on the dye, opposes the effect on the protein. On the basic side both protein and dye effect tend to diminish adsorption and hence the basic branch of the curve slopes very abruptly.

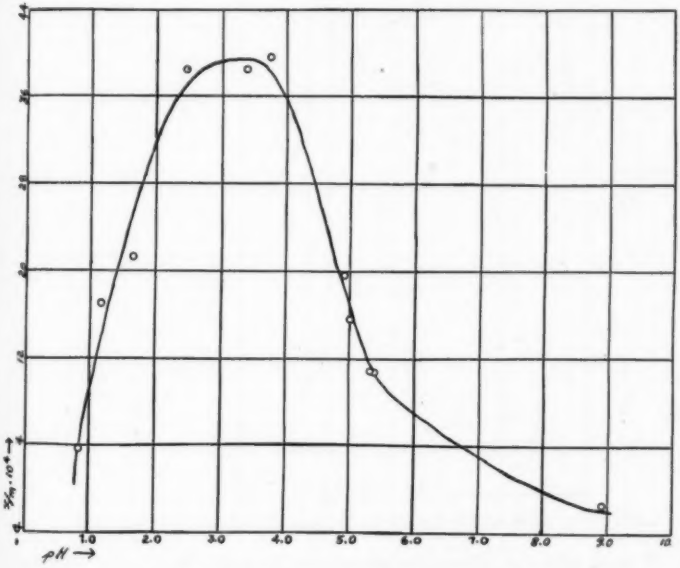


FIG. 5. Effect of pH on the adsorption of phenol red on gelatin.

The results obtained are seen to be in accord with these considerations as shown in Tables VIII to XI and Figs. 5 to 7 for gelatin (granular, undissolved), blood albumin, crystallized egg albumin, and fresh blood serum (pig). In the latter substance the results are limited to the more alkaline ranges as acidity causes precipitation. In the more alkaline ranges, a decided negative adsorption was obtained; *i.e.*, the concentration of dye in the ultrafiltrate was much greater than in the original solution, but due to

destruction of dye in this region, quantitative data are impossible. It should also be observed that combination takes place on either side of the isoelectric point in contradiction to the views of Loeb (15) and in accord with the more recent findings of Northrop and Kunitz (11) and Pauli and Schön (12).

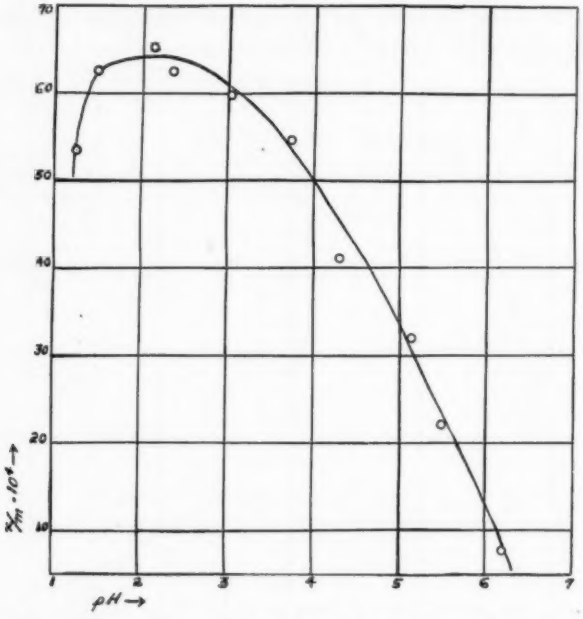


FIG. 6. Effect of pH on the adsorption of phenol red on egg albumin.

*Adsorption of Phenol Red by the Sera of Various Species.*

It has been previously found by Marshall and Vickers (8) that whereas phenol red, in the concentration used, was bound to an extent of 95 per cent by the blood serum of rabbits, only about 60 per cent was bound by that of the dog. In order to throw light on this variance in degree with which phenol red is bound by sera of various sources, its combination by whole serum and its separate protein constituents were studied.

The results with serum and albumin have already been given. Globulin adsorbs but feebly at the pH of the blood, as used in this investigation, compared with albumin under the same conditions. However, in more acid solution, globulin also adsorbs to quite an extent. Thus at pH 6.70, one-third of the dye was

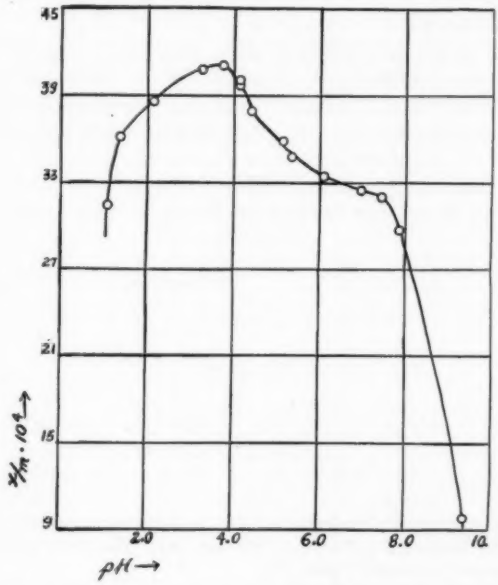


FIG. 7. Effect of pH on the adsorption of phenol red by blood albumin.

bound to an approximately 3 per cent solution of globulin in Ringer's solution. The amount of dye taken up from the same original concentration in various sera should, therefore, approximately, vary directly as the albumin content. Results obtained are shown in Table XII.

*Application to Kidney Function.*

The marked increase in adsorption with decreased pH may offer, in part at least, a physicochemical explanation of the removal of acid dyes from the blood stream and their acumulation in the

organs such as the liver and kidney. The latter are known (18) to be acidic as compared with the blood traversing them and hence there would be a tendency for the dye to accumulate in their cells in a concentration greater than that of the blood. Moreover, the higher albumin concentration of the cells will result in a similar concentration of the dye in the organs as compared with the fluid in the capillaries.

The use of phenolsulfonephthalein as a clinical agent in renal function tests has been criticized (19) for its unreliability due to extrarenal influences. As a result of this investigation one may clearly define the extent of two factors which will have an extra-

TABLE XII.

*Adsorption of Phenol Red by the Blood Sera of Various Species at Room Temperature.*

| Animal. | Albumin content. | Total dye bound. | Original dye concentration. |
|---|---|---|---|
| | *per cent* | *per cent* | *mg. per l.* |
| Rabbit | 4.8–6.7*(16) | 95 | 71 |
| Dog | 4.8 (17) | 75 | 74 |
| Pig | 4.3 (17) | 75 | 60 |
| Hen | 3.5 (17) | 54 | 79 |
| Duck | 3.2 (17) | 53 | 74 |
| Frog | 1.5† | 20 | 71 |

* There is considerable variation in the albumin content of rabbit serum under varying conditions of diet, age, etc. The value for adult rabbits approaches the higher figure (16).

† Determined refractometrically.

ordinary effect on the rate of elimination of the dye quite independent of any renal influences. Since the amount of dye held by the serum proteins, and, therefore, unfilterable, is determined by the albumin percentage composition of the blood and the pH of the latter, these two variables must be considered in addition to the excretion rate. Thus, in those pathological conditions in which there is a low albumin content of the blood with a correspondingly low albumin-globulin ratio, the amount of dye held in combination with the blood protein is low and this factor will greatly influence the excretion rate.

The complete mechanism of excretion in the organism is, to be

sure, quite complicated and, hence, no quantitative relation between excretion time and the factors considered here is to be expected due to other influencing factors. Nevertheless, the data on protein composition and phthalein output as found in the literature (20) show a striking agreement in this respect; *i.e.*, low albumin content and normal phthalein output are concomitant features of certain types of renal disturbances.

The magnitude of the effect of albumin concentration on the percentage of uncombined dye in the blood may be judged from the following tabulation. The percentage of total dye injected is assumed to be 6 mg. (21) into a plasma volume of 3 liters, and the approximate percentage of this amount bound, within the range of albumin concentrations met with in pathological conditions, are indicated.

| Albumin, *per cent* | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Uncombined dye | 84 | 68 | 52 | 36 | 20 | 4 |

SUMMARY.

1. The adsorption of phenol red on charcoal and a number of proteins has been quantitatively studied.

2. Adsorption curves for blood serum and hydrophilic systems are also given.

3. The effect of pH on the adsorption by these substances has been studied.

4. The effect of pH on adsorption by proteins is discussed and compared with the results obtained on charcoal.

5. Adsorption by blood sera of various animal species has been determined and compared.

6. Applications of the results to studies in renal function are indicated.

The problem of this investigation was suggested by and carried out under the supervision of Professor E. K. Marshall, Jr., for whose continued interest and help the author wishes to express his sincere gratitude.

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# FURTHER STUDIES ON THE CAUSE OF OPHTHALMIA IN RATS PRODUCED WITH DIETS CONTAINING VITAMIN A.

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(Received for publication, March 7, 1925.)

In a former paper (1) we described a type of ophthalmia in rats which resulted when the animals were restricted to a diet containing amounts of butter fat sufficient to furnish vitamin A to meet the physiological needs of the animals during growth. The ophthalmia was definitely shown to be produced by the character of the inorganic moiety of the diet. Ophthalmia was produced whether vitamin A was furnished by butter fat or by cod liver oil.

It was suggested in the paper referred to that an excess of chlorine and possibly an excess of sodium was the cause of this condition. This seemed to be the most plausible explanation. All the diets which had produced the ophthalmia contained excessive amounts of chlorine. We spoke of this ophthalmia as "salt ophthalmia," but Mori (2) concluded from a histological study of the eyes and related structures in our rats that the anatomical changes were identical with those seen in the ophthalmia produced by deprivation of vitamin A.

The invariable occurrence of ophthalmia in rats taking a diet containing 5 per cent of butter fat, when certain salt mixtures were used, and its prompt cure when the inorganic content of the food was changed, other components of the diet remaining unchanged, led us to undertake a careful study of the exact cause of the phenomenon.

In the studies reported we employed a diet consisting of wheat germ 3, wheat gluten 12, gelatin 10, egg albumin 10, agar-agar 2, salt mixture (No. 20) 4.1, $KH_2PO_4$ 1.7, dextrin 52.2, and butter

TABLE I.

*Percentage Composition of Diets Used in the Study of Salt Ophthalmia.*

| Ration No. | Yeast. | Wheat germ. | Maize. | Casein. | Wheat. | Rolled oats. | Wheat gluten. | Gelatin. | Egg albumin. | Agar-agar. | Salt mixture. No. | Salt mixture. Amount. | $KH_2PO_4$ | Dextrin. | Butter fat. | Sore eyes. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | | gm. | gm. | gm. | gm. | | |
| 3626 | 5 | | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 50.2 | 5 | + | |
| 3506 | | 3 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 52.2 | 5 | + | |
| 3579 | | 3 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 52.2 | 5 | + | |
| 3369 | | 3 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 52.2 | 5 | +‡ | |
| 3571 | | 3 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 52.2 | 5 | − | Had orange juice. |
| 3653 | | 3 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 52.2 | 5 | − | |
| 3771 | | 1 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 54.2 | 5 | + | |
| 3772 | | 2 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 53.2 | 5 | + | |
| 3462 | | 3 | | | | | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | 54.3 | 5 | − | |
| 3369 | | 3 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 52.2 | 5 | +‡ | |
| 3402 | | 3 | | 25 | | | | | | 2 | 21 | 3.9 | 1.7 | 59.4 | 5 | +‡ | |
| 3507 | | 10 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 45.2 | 5 | − | Very good growth. |
| 3606 | | 6 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 49.2 | 5 | − | Good growth. |
| 3595 | | | 15* | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 40.2 | 5 | − | |
| 3596 | | | 33* | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 22.2 | 5 | − | |
| 3651 | | 5 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 50.2 | 5 | − | |
| 3650 | 5 | | | | | | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | 52.3 | 5 | − | |
| 3580 | | 3 | | | | | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | 54.3 | 5 | − | |
| 3654 | | 4 | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 51.2 | 5 | − | |
| 3569 | | | | | 15 | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 40.2 | 5 | − | Good growth. |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 3569 | | * | | | 15 | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 51.2 | 5 | — | |
| | | | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 40.2 | 5 | — | Good growth. |
| 3570 | | | 15† | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 40.2 | 5 | — | Fair growth. |
| 3568 | | | | | | 15 | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 40.2 | 5 | — | |
| 3626 | 5 | | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 50.2 | 5 | +‡ | |
| 3751 | | 3 | | 18 | | | | | | 2 | 35 | 4.3 | | 67.7 | 5 | +‡ | |
| 3754 | | 3 | | | | | 12 | 10 | 10 | 2 | 34 | 5.6 | | 52.4 | 5 | — | |
| 3753 | | 3 | | | | | 12 | 10 | 10 | 2 | 36 | 5.2 | | 52.8 | 5 | — | |
| 3646 (Beef liver 3). | | | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 52.2 | 5 | — | |
| 3647 ( " " 6). | | | | | | | 12 | 10 | 10 | 2 | 20 | 4.1 | 1.7 | 49.2 | 5 | — | |

*White maize.
†Yellow maize.
‡ Eyes cured when salt mixture of diet was replaced by 3.7 gm. of salt mixture 185 per 100 gm. of ration.

fat 5 per cent respectively. Following the studies reported we have experimented with this diet, using the following substances in the amounts indicated as the sole source of vitamin B: wheat germ 1, 2, 3, 4, 5, 6, and 10 per cent respectively, yeast 5 per cent, beef liver 3 and 6 per cent, white maize 15 and 33 per cent, yellow maize 15 per cent, wheat 15 per cent, and rolled oats 15 per cent respectively, the diet remaining constant in composition except that the dextrin replaced the difference between the natural food added and the 3 per cent of wheat germ in the basal diet. The inorganic content of these diets was modified slightly by the mineral content of the materials added, but since the mineral content of the diet was high this could have modified the quality of the diet but little in any case. See Table I.

Diets in which 1, 2, or 3 per cent of wheat germ or 5 per cent of yeast was the sole source of vitamin B produced ophthalmia without exception. When five rats on a diet containing 3 per cent of wheat germ were given daily about 75 cc. of orange juice, ophthalmia did not occur. The same diet without the orange juice but containing 4, 5, 6, or 10 per cent of wheat germ as the sole source of vitamin B did not develop ophthalmia.

Rats fed 1 or 2 per cent of wheat germ grew poorly for about 6 weeks, then declined rapidly. Rats fed 3 per cent of wheat germ grew better in all cases than did those with 2 per cent, although considerable difference was observed in the rate of growth with different samples of germ. Those fed 5 per cent of yeast grew fairly well for 6 weeks and then failed rapidly, having developed sore eyes.[1] (See Chart 5, Lot 3626.) In Period 2, salt mixture XX was replaced by salt mixture 185, and the eyes returned to normal and the animals began to grow.

When the rats were fed beef liver 3 or 6 per cent, white maize 15 or 33 per cent, yellow maize 15 per cent, wheat 15 per cent, or rolled oats 15 per cent, they grew at approximately two-thirds the optimal rate and in no instance was ophthalmia observed. As with 4, 5, or 10 per cent of wheat germ the females were fertile in most instances, but the number of litters were too few, and the mortality of the young was high.

[1] We wish to express our thanks to Dr. M. H. Givens, of the Northwestern Yeast Company, who furnished the yeast used in these experiments.

Since the change from salt mixture XX, 4.1 per cent, to salt mixture 185, 3.7 per cent, led to recovery from the ophthalmia in every instance, we tried the effect of reducing the amount of salt mixture XX from 4.1 per cent to 2 per cent. On this diet the rats never developed ophthalmia when the diet contained 3 or more per cent of wheat germ, but they grew at only about two-thirds the normal rate. The females were fertile, each producing several litters. (See Chart 4, Lot 3580.) When 2 per cent of salt mixture XX was fed with the diet containing 5 per cent of yeast the animals grew at nearly the optimal rate for about 5 months, then began to decline and developed ophthalmia. (See Chart 5, Lot 3650.)

Since ophthalmia developed when 4.1 per cent of salt mixture XX was fed, but not when 2 per cent of it was added to the diet, we sought in the following manner to gain some information as to which of the salts in salt mixture XX caused the ophthalmia to develop. Using the basal diet of wheat germ 3, wheat gluten 12, gelatin 10, egg albumin 10, agar-agar 2, $KH_2PO_4$ 1.7, salt mixture (No. XX) 2.0, butter fat 5, and dextrin to make 100 per cent, we added in separate experiments the following salts: 1 per cent of NaCl (Lot 3572); 1.5 per cent of $CaCO_3$ (Lot 3573); 1 per cent of KCl (Lot 3574); 1 per cent of NaCl and 1.5 per cent of $CaCO_3$ (Lot 3575); 1 per cent of NaCl and 1 per cent of KCl (Lot 3576); 1 per cent of KCl and 1.5 per cent of $CaCO_3$ (Lot 3577); 1 per cent each of NaCl and KCl and 1.5 per cent of $CaCO_3$ (Lot 3578); and 1 per cent each of NaCl and KCl, 1.5 per cent of $CaCO_3$, and 0.2 per cent of MgO (Lot 3648). None of these animals developed ophthalmia although they were observed for about 13 months. The majority of these animals grew at about half the optimal rate and the females were fairly fertile. We were, therefore, unable to induce ophthalmia by excessive feeding of individual salts or with the several combinations described. These diets are given in detail in Table II.

Since we never observed ophthalmia in rats fed salt mixture 185, we tried the effect of supplementing 3.7 per cent of this mixture with 5 per cent of NaCl (Lot 3457); 5 per cent of $NaHCO_3$ (Lot 3458); 5 per cent of $CaCl_2$ (Lot 3459); 5 per cent of KCl (Lot 3460); 5 per cent of $CaCO_3$ (Lot 3536); 3 per cent of $CaCO_3$ (Lot 2946); and 0.3 per cent of MgO (Lot 3399). The basal

ration for this series was wheat germ 3, casein 18, salt mixture (No. 185) 3.7, agar-agar 2, butter fat 5 per cent, and dextrin to make 100 per cent. Ophthalmia developed only in Lot 3459 which received 5 per cent of $CaCl_2$, and these animals promptly recovered when the latter salt was omitted from the diet.

We also conducted experiments with this diet in which double the usual amount of salt mixture 185 was included in the diet; *viz.*, 7.4 per cent. In one case the diet contained 9 per cent of

TABLE II.

*Percentage Composition of Diets Used in Study of Salt Ophthalmia—Addition of Certain Salts.*

| Ration No. | Wheat germ. | Wheat gluten. | Gelatin. | Egg albumin. | Agar-agar. | Salt mixture. | | $KH_2PO_4$ | NaCl | KCl | $CaCO_3$ | MgO | Dextrin. | Butter fat. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | No. | Amount. | | | | | | | |
| | gm. | gm. | gm. | gm. | gm. | | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. |
| 3648 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | 1.0 | 1.0 | 1.5 | 0.2 | 50.6 | 5 |
| 3650 | (Yeast 5.) | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | | | | | 52.3 | 5 |
| 3580 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | | | | | 54.3 | 5 |
| 3577 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | | 1.0 | 1.5 | | 51.8 | 5 |
| 3578 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | 1.0 | 1.0 | 1.5 | | 50.8 | 5 |
| 3575 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | 1.0 | | 1.5 | | 51.8 | 5 |
| 3572 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | 1.0 | | | | 53.3 | 5 |
| 3573 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | | | 1.5 | | 52.8 | 5 |
| 3576 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | 1.0 | 1.0 | | | 52.3 | 5 |
| 3574 | 3 | 12 | 10 | 10 | 2 | 20 | 2.0 | 1.7 | | 1.0 | | | 53.3 | 5 |

The amount of each salt added gives in each case almost the same amount of the element which is contained in 4.1 gm. of salt mixture XX. This is not strictly true, for the amount is a little in excess of that which is contained in 4.1 gm. of salt mixture XX. In Lot 3648 there was a total salt content of 5.7 gm. whereas in salt mixture XX there was a total content of 4.1 per cent.

casein and in the other 18 per cent. No ophthalmia developed in either case.

We further attempted to intensify the effect of the salt content of the diet by reducing the protein content of the diet. Thus we fed wheat germ 3, casein 9, salt mixture (No. 185) 3.7, agar-agar 2, butter fat 5, and dextrin to make 100 per cent with the following salt additions: 5 per cent of KCl (Lot 3497); 5 per cent of $NaHCO_3$ (Lot 3495); 5 per cent of $CaCl_2$ (Lot 3496); 5 per cent of

$CaCO_3$ (Lot 3494); 2 per cent of MgO (Lot 3498); and 5 per cent of NaCl (Lot 3493). Those fed Diet 3496, containing the $CaCl_2$, developed ophthalmia. We fed the last described basal diet but with 18 per cent of casein, with only 1.8 per cent of salt mixture 185, supplemented with 5 per cent of NaCl (Lot 3470), and 5 per cent of $NaHCO_3$ (Lot 3471), but none of the rats developed ophthalmia.

Although the salts which we employed were C. P. reagents, we thought it possible that some impurity might be responsible for the eye trouble, so in another series we omitted salt mixture 185 and made only a single salt addition. In order to place an additional burden upon the animals the casein of the diet was reduced to 12 per cent. The salts employed in the different experiments were: NaCl 5 per cent (Lot 3501); $CaCO_3$ 5 per cent (Lot 3504); $NaHCO_3$ 5 per cent (Lot 3502); and KCl 5 per cent (Lot 3503). Although none of these animals grew appreciably they remained alive for 4 to 5 months and none of them developed ophthalmia. The diets for these series will be found in Table III.

## DISCUSSION.

It is apparent from the data reported that our early conjecture concerning the relation of excessive chlorine and possibly sodium in the diet to the development of ophthalmia was not well founded. Thus, in Lot 3470 there was 3.140 per cent of chlorine, and in Lot 3496 there was 3.305 per cent of chlorine. If taken alone these results would confirm the view that chlorine in high concentration is effective in inducing ophthalmia, but in our studies reported earlier we regularly induced ophthalmia with salt mixture XX, which furnished only 0.779 per cent of this element in the diet. Other illustrations of the appearance of ophthalmia in animals receiving much less chlorine than was furnished by 5 per cent of NaCl in the diet will be found in Table IV of our previous paper (1).

The data in Tables III and V also show that excessive sodium cannot be the sole cause of the ophthalmia, since Lots 3471, 3495, and 3458 all received 5 per cent of sodium bicarbonate but none of the animals developed ophthalmia. An inspection of these tables will likewise show that excess of calcium, magnesium, or potassium is not capable of inducing the eye condition. Low

TABLE III.

*Percentage Composition of Diets Used in the Study of Ophthalmia. Salt Mixture 185 as a Basis.*

| Ration No. | Wheat germ. | Casein. | Salts 185. | Agar-agar. | Dextrin. | NaCl | $NaHCO_3$ | KCl | $CaCl_2$ | $CaCO_3$ | MgO | Butter fat. | Remarks. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | |
| 3471 | 3 | 18 | 1.8 | 2 | 65.2 | | 5 | | | | | 5 | |
| 3470 | 3 | 18 | 1.8 | 2 | 65.2 | 5 | | | | | | 5 | One developed edema. |
| 3500 | 3 | 9 | 7.4 | 2 | 73.6 | | | | | | | 5 | |
| 3499 | 3 | 18 | 7.4 | 2 | 64.6 | | | | | | | 5 | |
| 3553 | 5 | 18 | 5.0 | 2 | 65.0 | | | | | | | 5 | |
| 3497 | 3 | 9 | 3.7 | 2 | 72.3 | | | 5 | | | | 5 | |
| 3495 | 3 | 9 | 3.7 | 2 | 72.3 | | 5 | | | | | 5 | |
| 3496 | 3 | 9 | 3.7 | 2 | 72.3 | | | | 5 | | | 5 | These developed sore eyes. |
| 3494 | 3 | 9 | 3.7 | 2 | 72.3 | | | | | 5 | | 5 | Not in good condition but no sore eyes. |
| 3498 | 3 | 9 | 3.7 | 2 | 75.3 | | | | | | 2 | 5 | |
| 3535 | 3 | 9 | 3.7 | 2 | 77.3 | | | | | | | 5 | |
| 3493 | 3 | 9 | 3.7 | 2 | 72.3 | 5 | | | | | | 5 | |
| 3446 | 3 | 18 | 3.7 | 2 | 67.3 | | | 1 | | | | 5 | |
| 3444 | 3 | 18 | 3.7 | 2 | 65.3 | 2 | | 1 | | | | 5 | |
| 3445 | 3 | 18 | 3.7 | 2 | 66.3 | 2 | | | | | | 5 | |
| 2946 | 4 | 18 | 3.7 | 2 | 59.3 | | | | | 3 | | 10 | |
| 3536 | 3 | 18 | 3.7 | 2 | 63.3 | | | | | 5 | | 5 | |
| 3458 | 3 | 18 | 3.7 | 2 | 63.3 | | 5 | | | | | 5 | |
| 3457 | 3 | 18 | 3.7 | 2 | 63.3 | 5 | | | | | | 5 | |
| 3459 | 3 | 18 | 3.7 | 2 | 63.3 | | | | 5 | | | 5 | Sore eyes developed. Cured by leaving out $CaCl_2$ (*i.e.* on salt mixture 185). |
| 3447 | 3 | 18 | 3.7 | 2 | 65.3 | 3 | | | | | | 5 | |
| 3460 | 3 | 18 | 3.7 | 2 | 63.3 | | | 5 | | | | 5 | |
| 3399 | 3 | 18 | 3.7 | 2 | 68.0 | | | | | | 0.3 | 5 | |
| 3554 | 5 | 18 | 3.7 | 2 | 65.3 | | | | | 1 | | 5 | |
| 3453 | 3 | 18 | | 2 | 72.0 | | | | | | | 5 | |
| 3501 | 3 | 12 | | 2 | 73.0 | 5 | | | | | | 5 | |
| 3502 | 3 | 12 | | 2 | 73.0 | | 5 | | | | | 5 | |
| 3503 | 3 | 12 | | 2 | 73.0 | | | 5 | | | | 5 | |
| 3504 | 3 | 12 | | 2 | 73.0 | | | | | 5 | | 5 | |

protein feeding in the experiments described did not appear to predispose the animals to ophthalmia.

Our experience with varying the content of vitamin B in the food affords a suggestion as to a possible explanation for the development of ophthalmia in certain of our animals. Ophthalmia

TABLE IV.

*Percentage Composition of Salt Mixtures.*

| Mixture No. | NaCl | Anhydrous $MgSO_4$. | $NaH_2PO_4 + H_2O$ | $K_2HPO_4$ | $CaH_4(PO_4)_2 + H_2O$ | Ferric lactate. | Calcium lactate. | $CaCO_3$ | KCl | $NaHCO_3$ | MgO | $FeSO_4 + 7H_2O$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. |
| 185 | 0.173 | 0.266 | 0.347 | 0.954 | 0.540 | 0.118 | 1.300 | | | | | |
| XX | 0.5 | | | | | | | 1.5 | 1.0 | 0.7 | 0.2 | 0.2 |
| XXI | 0.5 | | | | | | | 1.5 | 1.0 | 0.7 | | 0.2 |

It should be noted that salt mixture XX contained no organic acid radicals, yet we secured good growth records with it in certain of our experiments.

TABLE V.

*Composition of Salt Mixtures.*

| Mixture No. | Amount of mixture. | Na | Cl | P | Mg | Ca | S | Fe | K |
|---|---|---|---|---|---|---|---|---|---|
| | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. | gm. |
| 185 | 3.7 | 0.126 | 0.105 | 0.379 | 0.053 | 0.255 | 0.071 | 0.023 | 0.428 |
| XX | 2.0 | 0.190 | 0.375 | | 0.059 | 0.299 | 0.011 | 0.019 | 0.255 |
| XX | 4.1 | 0.389 | 0.779 | | 0.120 | 0.600 | 0.023 | 0.040 | 0.524 |
| XXI | 3.9 | 0.389 | 0.779 | | | 0.600 | 0.023 | 0.040 | 0.524 |

18 gm. of casein contain 0.150 gm. P.

$KH_2PO_4$ contains 28.68 per cent K.

" " 22.79 " " P.

1.7 " " 0.487 gm. K.

1.7 " " 0.387 " P.

developed in those animals fed diets containing 1, 2, and 3 per cent of wheat germ, as it likewise did in those fed 5 per cent of yeast as the sole source of vitamin B when 4.1 per cent of salt mixture XX was employed. On the other hand, when 4, 5, 6, or 10 per cent of wheat germ was included in the diet ophthalmia did not develop. It is further significant that when 3 per cent

of wheat germ was fed there was considerable variation in the length of time necessary to induce the condition. We repeated the experiment with five groups of animals at different seasons and with different samples of germ and observed a variation of from 6 weeks to 5 months before the eyes appeared abnormal. None of the animals failed to develop the ophthalmia in time. We never saw this condition when 4 or more per cent of wheat germ was fed.

When we reduced the amount of salt mixture XX to 2 per cent of the food, ophthalmia did not develop even when only 3 per cent of germ was included in the diet. On this diet rats grew at about two-thirds the normal rate and the females produced several litters of young each (See Chart IV, Lot 3580). On the other hand, with either 2 or 4.1 per cent of the salt mixture and 5 per cent of yeast the animals developed sore eyes, but it required much longer when they were fed 2 per cent than when fed 4 per cent. (See Chart 5.) Repetition of the experiments confirmed these observations.

The most plausible explanation for these remarkable results appears to be found in the interrelation between the appropriateness of the relationship between certain of the nutritive principles in these dietaries. There is no doubt that feeding too much of a salt mixture constitutes a physiological burden for the animal, but if all other components of the diet are present in nearly optimal amounts the body may be able to sustain itself in a manner which appears on casual inspection to be normal. If, however, the experiment is extended and the capacity of the organism to sustain the burden of the unfavorable dietary without showing premature signs of deterioration is observed, it will, we are convinced by many observations, be apparent in its life history that an insidious influence is at work to undermine vitality. If, now, a second factor is made unsatisfactory, signs of failure appear earlier than would have been the case had but a single component of the diet been faulty. We have in the experiments reported observed a most unexpected appearance of ophthalmia under conditions of nutritive disturbance entirely different from those which have hitherto been known to induce it; *viz.*, lack of vitamin A. It appears that in the experiments which we are now discussing, the determining factor as to whether or not the ophthalmia

would appear was the content of vitamin B in the diet This was not, however, the primary cause, but only acted as an accelerating influence in determining whether and when the effects of an unfavorably constituted inorganic intake would manifest itself.

In none of the scores of rats which we have restricted to diets deficient in vitamin B have we observed ophthalmia, but these diets were all fairly well constituted except with respect to this vitamin. It seems plausible to interpret our data to mean that when the content of the diet in vitamin B is very near the minimum essential for the maintenance of the rat, the animals are injured by salt mixtures of unfavorable composition, the injury manifesting itself, among other ways, in the appearance of ophthalmia. When vitamin B is supplied more liberally by the inclusion of more wheat germ, the animals are able to withstand better the unfavorable effects of an unappropriate mineral content in their dietary.

There are certain difficulties in accepting this interpretation. Thus, 5 per cent of yeast should, according to all recorded observations, supply enough of vitamin B to support optimal growth and maintain optimal vitality in the rat. Yeast is generally believed to be somewhat richer in vitamin B than is wheat germ, yet 4 or 5 per cent of germ prevented ophthalmia and the animals grew fairly well, whereas 5 per cent of yeast permitted ophthalmia to develop and the growth curves were not so good. In a series of experiments in which the standard diet was fed but in which the sole source of vitamin B was 15 per cent of white maize, or yellow maize, of wheat, or of rolled oats, respectively, or 3 per cent of steamed, dried liver, the animals grew well and never had sore eyes. It seems improbable, judging from existing data, that the amounts of cereal grains included in certain of our diets should furnish more vitamin B than did 3 per cent of wheat germ or 5 per cent of yeast. We have repeated the more significant of the experiments, in some cases as many as five times, and are convinced that the results can be secured at any time by reproducing the conditions of our experiments. We find greater difficulty in interpreting these results than any other we have met with in our experience.

A possible explanation of the phenomena described is that vitamin B is not one substance but contains two or more principles.

XUM

Although this view has been expressed by others no one has brought forth the slightest evidence to support it. It may be possible that yeast contains both of these principles but in different proportions than are contained in wheat germ, and that on this basis one may explain the inferiority of yeast as contrasted with germ in protecting the rats against our faulty salt mixtures. For example, one might speculate that there are two nutritive essentials composing vitamin B, which may be designated as $y$ and $z$. In yeast $y$ may be present in lesser amount than $z$, and so when the minimum amount of yeast is fed which can support growth in the rat when the rest of the diet is well constituted, $y$ is the limiting factor, and $z$ is fed more liberally than $y$. In wheat germ $y$ and $z$ might be present in more favorable proportions (in certain samples) than in yeast, so that when fed to the amount of 4 per cent of the diet, likewise well constituted, neither $y$ nor $z$ would be provided in minimal amount, hence the physiological stability would be greater in this case than when the minimum amount of yeast is fed. When the diet is appropriately constituted, little or no difference might be demonstrable between the values of yeast and germ as sources of vitamin B ($y$ and $z$), but the superiority of one over the other might be demonstrable when the diet is made unfavorable through the addition of inappropriate salt mixtures.

An illustration of the complicated situations which one sometimes has to analyze in nutrition studies is seen in our procedure for differentiating between the nutritive qualities of cod liver oil and of butter fat in relation to their special function in regulating calcification of the bones (3). If a diet is satisfactory in respect to its calcium and phosphorus content, and to all other factors except that which may be provided by certain fats (vitamin D), the bones may develop in essentially a normal manner when either butter fat or cod liver oil provides vitamins A and D. On the other hand, if the same diet is depleted in calcium but contains the optimal amount of phosphorus, calcification will take place in a fairly normal manner when cod liver oil, but not when butter fat, is added. This is understandable in the light of our knowledge that there are two indispensable substances in each of these fats; *viz.*, vitamins A and D. Butter fat contains relatively much vitamin A and little vitamin D, whereas cod liver oil is rich in

both. The needs of the body for vitamin D seem to be satisfied by a small intake of this substance when conditions are otherwise optimal for calcification, and are progressively greater the more the content of the diet deviates from the optimal in respect to its calcium and phosphorus content.

Another peculiar result met with in nutrition studies is illustrated by the conditions met with in testing fats for their content of vitamin A. Thus, when young rats produced from our stock ration are confined to a diet of rolled oats 40, casein 5, NaCl 1, $CaCO_3$ 1.5, and dextrin 52.5, they do not grow appreciably and develop ophthalmia within 4 to 6 weeks. If given 2 per cent of butter fat or of cod liver oil the eyes become normal in 5 to 10 days. If, however, either of the following diets are fed, *viz.*, rolled oats 60, casein 18, NaCl 1, $CaCO_3$ 1.5, and dextrin to 100; or rolled oats 60, wheat gluten 10, gelatin 10, NaCl 1, $CaCO_3$ 1.5, and dextrin to 100, the animals grow for 8 to 12 weeks and then begin to decline, but have not developed ophthalmia at this time. They may not show the eye condition for 3 or 4 weeks longer. When they finally develop ophthalmia due to deprivation of vitamin A they are so enfeebled that recovery is difficult, and in many cases cannot be induced by feeding vitamin A in abundance. The cause of this difference appears to be that a liberal protein supply tends to promote well-being and stimulate growth, even when the animals are daily growing more depleted with respect to vitamin A. They seem to be protected against ophthalmia to a point where deprivation for vitamin A has wrought greater damage than is the case where ophthalmia appears early when the diet is poor in protein. Certain it is that their ability to recover is markedly less when the deprivation has extended over the longer period made possible by high protein feeding.

## SUMMARY.

1. Further studies are described on the conditions under which ophthalmia appears in rats taking a diet containing an adequate amount of vitamin A when excessive amounts of certain salts are contained in the diet.

2. It is demonstrated that the ophthalmia cannot be induced by feeding excessive amounts of any one element or ion. The total amount of a salt mixture and its composition, which is included,

may be the determining factor as to whether ophthalmia will develop.

3. Conditions are described with respect to the inorganic salt content of the food under which the determining factor as to whether ophthalmia will appear is the amount of wheat germ added as a source of vitamin B.

4. Yeast, when serving as the sole source of vitamin B, was decidedly less effective than equal amounts of wheat germ in protecting rats against the detrimental effects of excessive salt feeding.

5. Our former surmise that the ophthalmia was due to excessive amounts of chlorine or possibly of sodium in the diet is proven untenable. A discussion is presented of certain of our experiences which illustrate the significance of some interrelations between the appropriateness of relationship between certain of the nutritive principles in the dietaries. The results described are not to be accounted for by any existing views on the relation between dietary defects and pathological changes. As a possible explanation of the experimental results recorded the dual nature of vitamin B is suggested.

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Lot 3869
Chart 1, Lot 3506
Ration
XUM

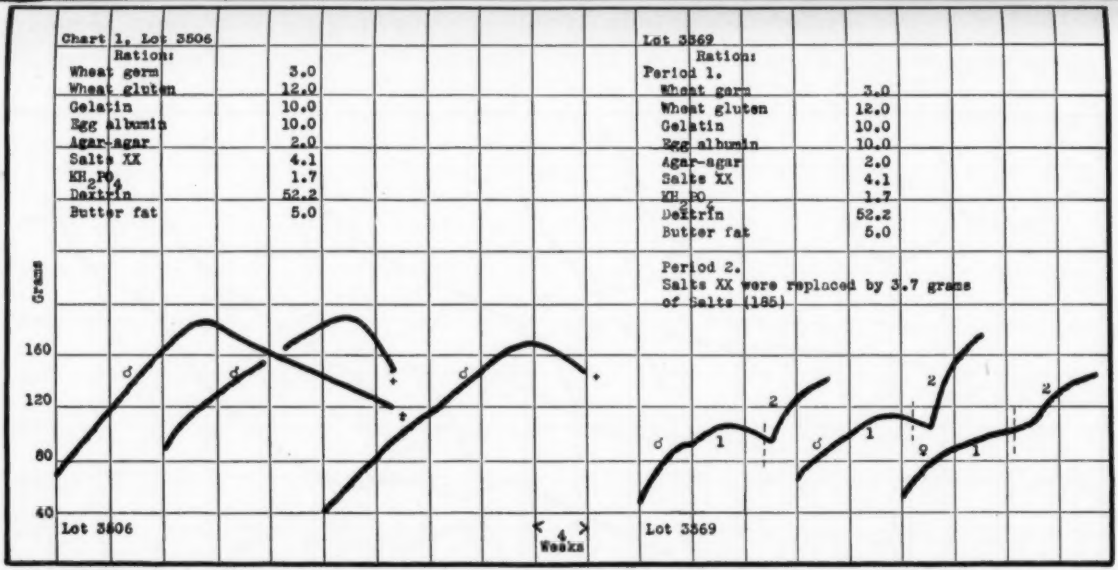


CHART 1, LOT 3506. These are typical growth curves of animals confined to Diet 3506. As a rule ophthalmia appears after decline in weight has begun.

LOT 3369. These curves illustrate the effect of changing the salt mixture in the diet on which ophthalmia has been induced by salt mixture XX. In Period 1 salt mixtureXX was fed. After ophthalmia developed the diet was made up anew with 3.7 per cent of salt mixture 185 in place of 4.1 per cent of salt mixture XX. The eyes returned to a normal condition as a result of this change. The two sets of curves on this chart show the extent of variation of growth which we have seen in different experiments on the same food formula. This was apparently due to variation in the vitamin B content of wheat germ.

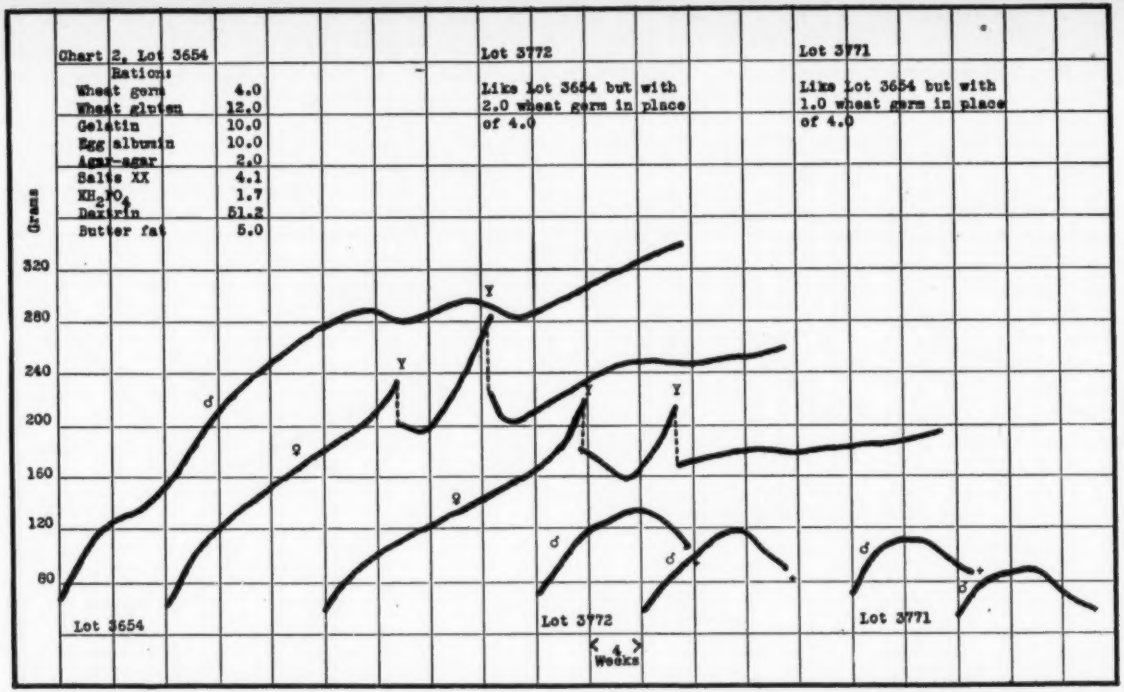


Chart 2, Lots 3771 and 3772. These are representative growth curves of young rats on our basal diet containing 1 and 2 per cent respectively of wheat germ. After a period of growth the animals always developed ophthalmia when salt mixture XX was included in the diet. This was not due to partial deprivation of vitamin B but to more complicated causes.

Lot 3654. These curves show the remarkable difference in the rate and extent of growth on the basal diet when 4 per cent of wheat germ was included in the diet. These curves should be compared with those in Chart 1, where 3 per cent of germ was fed.

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Lot 3654. These curves show the remarkable difference in the rate and extent of growth on the basal diet when 4 per cent of wheat germ was included in the diet. These curves should be compared with those in Chart 1, where 3 per cent of germ was fed.

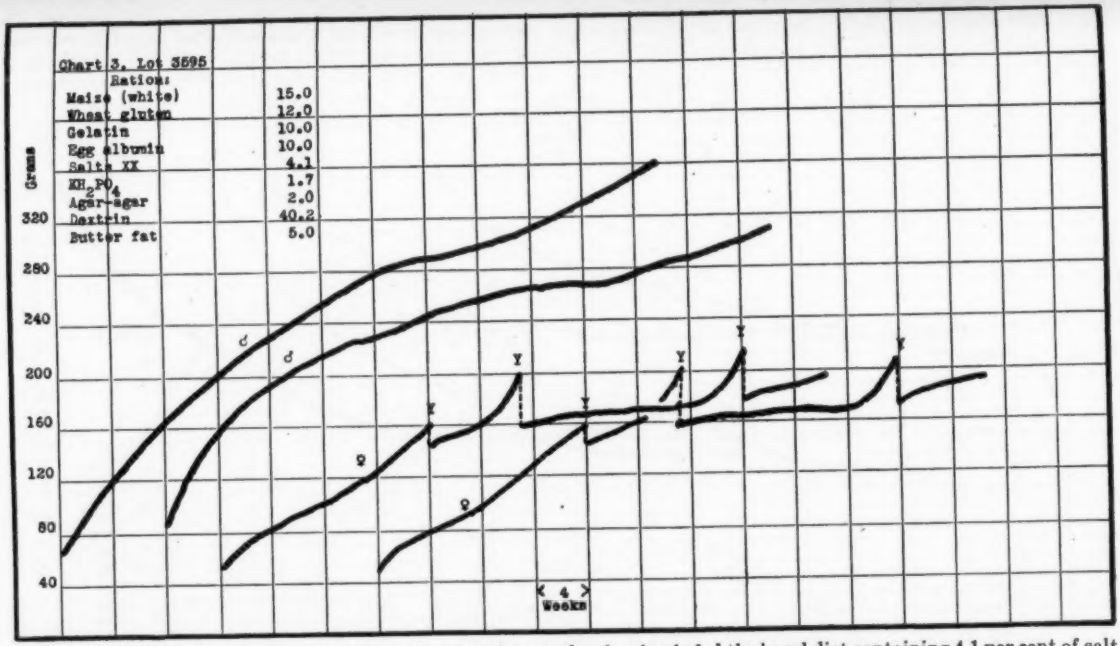


Chart 3, Lot 3595. These curves show the growth records of animals fed the basal diet containing 4.1 per cent of salt mixture XX and deriving its vitamin B entirely from 15 per cent of white maize. These should be compared with those in Chart 1, in which the vitamin B was supplied by 3 per cent of wheat germ. Lot 3595 never developed ophthalmia. Similar results were obtained with yellow maize.

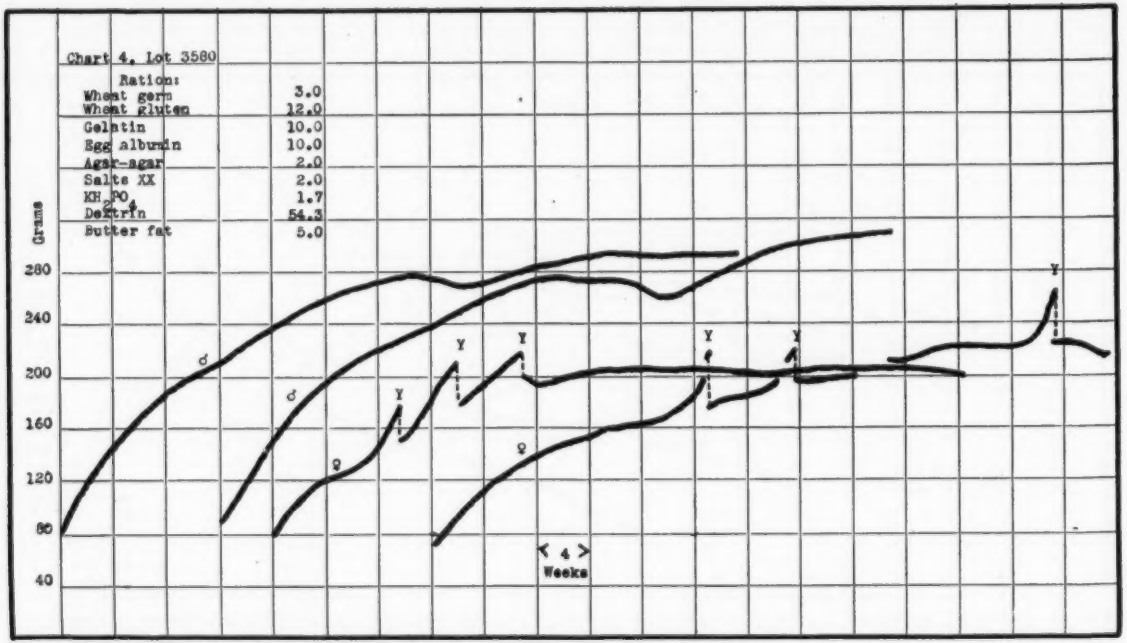


CHART 4, LOT 3580. These curves illustrate the amount of growth secured with the basal diet containing but 3 per cent of wheat germ, and but 2 per cent of salt mixture XX. Rats cannot thrive on this amount of wheat germ when 4.1 per cent of salt mixture XX is contained in their diet, as is shown in Chart 1.

CHART 4, LOT 3580. These curves illustrate the amount of growth secured with the basal diet containing but 3 per cent of wheat germ, and but 2 per cent of salt mixture XX. Rats cannot thrive on this amount of wheat germ when 4.1 per cent of salt mixture XX is contained in their diet, as is shown in Chart 1.

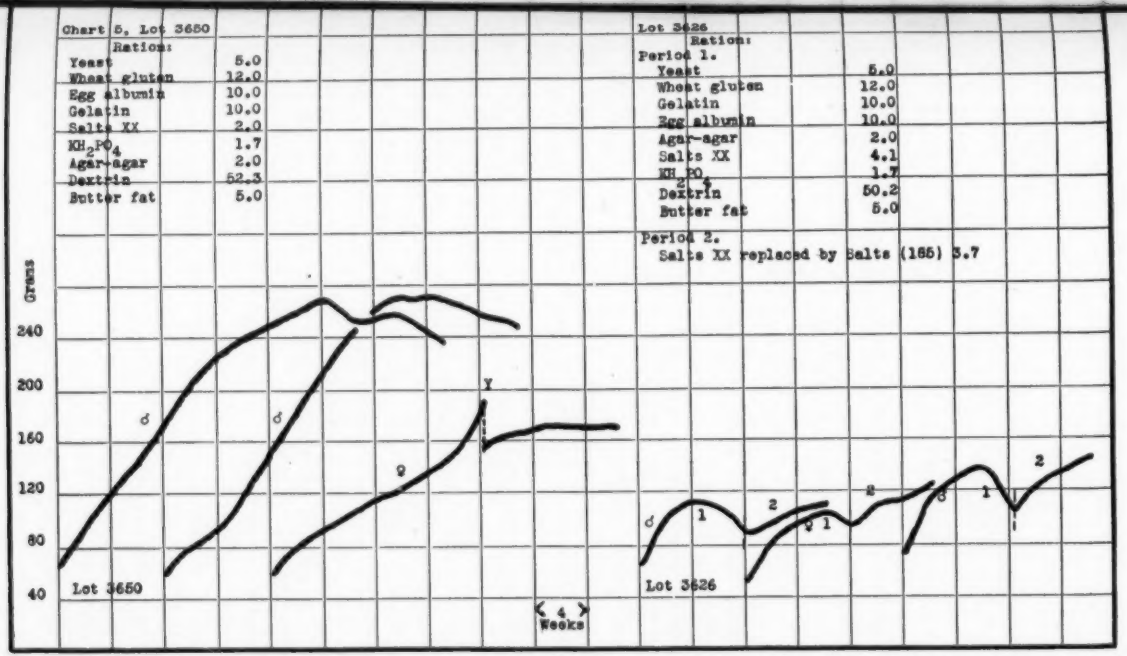


CHART 5, LOT 3626. Period 1. These rats were fed 5 per cent of yeast with the basal diet containing 4.1 per cent of salt mixture XX. A comparison of these records with those of Lot 3654, Chart 2, reveals the fact that our yeast was not so rich in vitamin B, or else some factor present in wheat germ which we do not now understand in some manner protected the animals against the detrimental effects of taking the basal diet containing 4.1 per cent of salt mixture XX. The animals in Lot 3626 all developed ophthalmia, and recovered when salt mixture XX was replaced by 3.7 per cent of salt mixture 185.

LOT 3650. This lot shows the rate of growth secured when 5 per cent of yeast was employed to furnish vitamin B, but with only 2 per cent of salt mixture XX in the food mixture. These rats all developed sore eyes after being on the diet about 5½ months.

# THE ANTIRACHITIC VALUE OF IRRADIATED CHOLESTEROL AND PHYTOSTEROL.

## II. FURTHER EVIDENCE OF CHANGE IN BIOLOGICAL ACTIVITY.

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In previous communications (1) it has been reported that various foods which are inactive or which in a natural state possess no antirachitic virtue can be rendered active in this respect by means of ultra-violet irradiation. Similar results have been reported by Steenbock(2) and his coworkers. It has been shown, furthermore, that this antirachitic factor is confined to the non-saponifiable fraction of these substances, and that it resides largely or entirely in the cholesterol or the phytosterol which they contain.

These investigations have been continued and extended. It would seem that any substance which contains cholesterol or phytosterol can be activated to a greater or less extent by means of irradiation and that there will be differences merely in the degree of potency which is developed. In a previous communication it was stated that 0.5 cc. of irradiated fluid milk was insufficient to protect rats from rickets. Subsequent tests with dry milk have demonstrated that this food can readily be endowed with antirachitic potency, so that the standard rickets-producing ration containing 5 per cent of dry milk can be converted to a rickets-protective ration, if the dry milk has been irradiated before having been incorporated with the flour and salt mixture. This result is illustrated in Table I. Probably it will be found that if larger amounts of irradiated fluid milk are fed protection likewise will be brought about. Our previous tests showed that patent wheat flour can be changed to an antirachitic food by means of irradiation with the quartz mercury vapor lamp. The same is true of



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spinach. Moreover, it was found that the activated spinach does not lose this property on subsequent boiling for a period of ½ hour, that when the animals are given 10 gm. per capita of this cooked spinach they are regularly protected from rickets (Table II). Preliminary tests have shown that irradiated vegetables and dry milk possess curative value in the rickets of infants, as judged by the radiographic picture and the increase of inorganic phosphorus in the blood. Irradiated oleic acid fed in 0.25 cc. amounts as well as egg phosphatide, fed in the same amounts, did not prevent the occurrence of rickets.

TABLE I.

*Feeding Experiments with Irradiated and Non-Irradiated Dry Milk.*

| Rat No. | Weight. | Diet. | Rickets. | | | Blood P (inorganic). |
|---|---|---|---|---|---|---|
| | | | Radiographic. | Macroscopic. | Microscopic. | |
| | *gm.* | *per cent* | | | | *mg. per cent* |
| 7314 | 56–76 | Dry milk (*irradiated* ½ hr. at | No. | No. | Negative. | 4.92 |
| 7315 | 40–64 | 1 ft.)..... 5.0 | " | " | " | |
| 7316 | 60–88 | Flour.....90.0 | " | " | " | |
| | | Salt mixture..... 5.0 | | | | |
| 7318 | 50–76 | Dry milk (non- | Moderate. | Moderate. | Moderate. | 1.44 |
| 7319 | 40–64 | irradiated).... 5.0 | " | " | Marked. | |
| 7320 | 56–56 | Flour.....90.0 | " | " | Moderate. | |
| 7321 | 60–80 | Salt mixture..... 5.0 | " | " | " | |

Experiments have been continued with cholesterol. An exposure of somewhat more than 2 minutes was required to activate a 1 per cent aqueous suspension of cholesterol when the burner was placed at a distance of 1 foot and when each rat was given 0.25 cc. of this suspension daily. Irradiated cholesterol was also able to protect against rickets when given by the subcutaneous route; 0.1 and 0.25 cc. of a watery suspension were injected daily as illustrated in Table III. The injection was made into the back and the site of puncture was swabbed with picric acid in order to prevent the possibility of the rats obtaining any of the test material by mouth. Each rat was kept in a separate cage. This result is of interest, showing as it does that it is unnecessary to give the

TABLE II.

*Feeding Experiments with Irradiated Spinach Subsequently Cooked.*

| Rat No. | Weight. | Rickets-producing diet. | Spinach. | Rickets. | | | Blood P (inorganic). |
|---|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. | |
| | *gm.* | | | | | | *mg. per cent* |
| 7298 | 64–89 | Low phosphorus. No. 84. | 10 gm. *irradiated* ½ hr. at 1 ft. and boiled ½ hr. | No. | No. | Negative. | 3.24 |
| 7299 | 54–60 | | | " | " | " | |
| 7300 | 34–58 | | | " | " | " | |
| 7301 | 56–84 | | | " | " | " | |
| 7302 | 50–60 | " " | 10 gm. (non-irradiated) boiled ½ hr. | Moderate. | Moderate. | Slight. | 1.83 |
| 7303 | 64–74 | | | Slight. | " | " | |
| 7304 | 32–60 | | | Marked. | Marked. | Marked. | |
| 7305 | 48–64 | | | Moderate. | " | Moderate. | |

TABLE III.

*Irradiated Cholesterol Given Subcutaneously.*

| Rat No. | Weight. | Rickets-producing diet. | Amount injected. | Rickets. | | | Blood P (inorganic). |
|---|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. | |
| | *gm.* | | | | | | *mg. per cent* |
| 7351 | 34–40 | Low phosphorus. No. 84. | 0.25 cc. 1 per cent watery suspension cholesterol *irradiated* ½ hr. at 1 ft. | No. | No. | Negative. | |
| 7352 | 40–50 | | | " | " | " | |
| 7353 | 50–49 | | | " | " | " | |
| 7419 | 40–44 | " " | 0.1 cc. 1 per cent watery suspension cholesterol *irradiated* ½ hr. at 1 ft. | " | " | | 5.72 |
| 7420 | 40–40 | | | " | " | | |
| 7421 | 50–52 | | | " | " | | |
| 7422 | 34–44 | | | " | " | | |

activated cholesterol by mouth; it functions when given parenterally. It has not been definitely established whether cod liver oil is effective when given subcutaneously. Zucker and Matzner (3), however, failed to bring about a cure in rats by injections of the active principle of cod liver oil dissolved in olive oil or in glycerol.

Some time ago we reported (4) that the radiations which are effective in protecting against rickets are those which are about 300 $m\mu$ or less in wave-length. A series of experiments was carried out with selective filters and it was determined that the intense line of the mercury vapor spectrum 302 $m\mu$ in length conferred definite protection and that the 313 $m\mu$ waves exerted probably a feeble action in this respect. These same filters were interposed between the mercury vapor burner and the 1 per cent aqueous suspension of cholesterol. In order to ascertain to what degree filtration interfered with the activation of the cholesterol, three filters were used as may be noted in Table IV. The first, G-586-A, transmits a considerable intensity of the 313 $m\mu$ line and a small intensity of the 302 $m\mu$ line. When this filter was interposed it was found that activation was somewhat interfered with, that the cholestrol irradiated in this way was unable to confer complete protection. Another filter which was used was Pyrex glass also manufactured by the Corning Company. This filter allows the transmission of shorter rays extending down to 289 $m\mu$ or even 280 $m\mu$. The cholesterol irradiated with the interposition of the Pyrex glass was rendered antirachitic and the rats which received this cholesterol had a comparatively high percentage of inorganic phosphorus in their blood. It will be noted, however, that there was one exception to this rule, that one animal developed moderate rickets. A third filter was one manufactured by the Hanovia Chemical Company, a new gas filter (No. 25–26) which contains chlorine and bromine and has the exceptional quality of transmitting less than 0.1 per cent of radiations between 290 $m\mu$ and the visible blue. It allows the passage of 90 per cent of waves 265 and 254 $m\mu$ in length and extends as far down in the spectrum as 200 $m\mu$. In other words, it affords the opportunity of testing the activity of the short ultra-violet radiations which have been segregated from the longer ultra-violet waves. When this filter was used the rats were protected, as shown by the radiographs, by the microscopic examination of the costochondral junctions

and the inorganic phosphorus content of the blood. It is evident, therefore, that the cholesterol reacted to these radiations in the far ultra-violet. Surveying these results carried out with filters, it would seem that cholesterol is activated by ultra-violet radiations of approximately the same wave-lengths as those which have

TABLE IV.

*Feeding Experiments with Cholesterol Irradiated through Filters.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | | Blood P (inorganic). |
|---|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. | |
| | *gm.* | | | | | | *mg. per cent* |
| 7371 | 36–36 | Low phosphorus. No. 84. | 0.25 cc. 1 per cent watery suspension cholesterol *irradiated* 1 hr. at 9 in. through G-586-A filter. | Slight. | No. | Slight. | |
| 7372 | 40–44 | | | " | Very slight. | " | |
| 7373 | 40–34 | | | No. | " | Very slight. | |
| 7411 | 45–46 | " " | 0.25 cc. 1 per cent watery suspension cholesterol *irradiated* ½ hr. at 1 ft. through Pyrex filter. | Moderate. | Moderate. | | 4.48 |
| 7412 | 53–54 | | | No. | No. | | |
| 7413 | 30–40 | | | " | " | | |
| 7414 | 40–45 | | | " | " | | |
| 7415 | 42–48 | " " | 0.25 cc. 1 per cent watery suspension cholesterol *irradiated* ½ hr. at 1 ft. through No. 25–26 gas filter. | " | " | | 4.25 |
| 7416 | 40–40 | | | " | " | | |
| 7418 | 44–50 | | | Slight. | Very slight. | | |

been found to be specific in rickets where animals are subjected to direct irradiation.

As is well known, rickets can be produced in rats by a ration which is either low in phosphorus or low in calcium. Our previous experiments with irradiated substances have been carried out with a diet low in phosphorus, the No. 84 Sherman-Pappenheimer

ration. Cod liver oil, as well as direct ultra-violet irradiation, is able to protect animals which are fed on either type of diet. It seemed of interest to ascertain whether the same holds good for irradiated cholesterol. Such was found to be the case as is shown in Table V. This diet leads to a lowered percentage of calcium in the blood and to a marked increase in nervous irritability. The condition is probably comparable to the tetany of infants, so that,

TABLE V.

*Feeding Irradiated and Non-Irradiated Cholesterol to Rats on a Low Calcium Rickets-Producing Ration.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | | Blood P and Ca (inorganic). |
|---|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. | |
| | *gm.* | | | | | | *mg. per cent* |
| 7343 | 40–50 | Low | 0.25 cc. watery suspension cholesterol *irradiated* ½ hr. at 1 ft. | No. | No. | No. | |
| 7344 | 40–44 | calcium. | | " | " | " | |
| 7345 | 50–60 | No. 85 | | " | " | " | |
| 7346 | 50–60 | C. | | " | " | " | |
| 7347 | 44–50 | " " | 0.25 cc. 1 per cent watery suspension cholesterol. | " | Very slight. | " | |
| 7348 | 40–39 | | | " | " " | Slight. | |
| 7382 | 70–60 | | | " | No. | Moderate. | P...5.88 |
| 7383 | 60–64 | | | " | " | " | Ca. 7.2 |
| 7384 | 52–54 | | | " | " | Slight. | |
| 7385 | 60–64 | | | " | " | " | |

by analogy, one may expect irradiated foods or cholesterol to be protective or curative not only in the rickets but also in the tetany of infants. The results of feeding a low calcium diet to rats must be judged by the microscopic picture, as the radiographic films and the macroscopic lesions frequently fail to demonstrate any evidence of rickets. The histologic lesion is entirely different from that which results from the low phosphorus diet and evidently is a nutritional disorder of a quite different nature.

In view of the fact that ultra-violet light is a specific in the cure and protection of rickets, and that these radiations do not penetrate the skin, an experiment was devised to determine whether

TABLE VI.

*Feeding Experiments with Irradiated and Non-Irradiated Skin.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | | Blood P (inorganic). |
|---|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. | |
| | gm. | | | | | | mg. per cent |
| 7388 | 60–60 | Low | 1 gm. human skin | No. | No. | | 2.97 |
| 7389 | 74–80 | phos- | *irradiated* | " | " | | |
| 7390 | 70–80 | phorus. | ½ hr. at 1 ft. | " | " | | |
| 7391 | 60–70 | No. 84. | | " | " | | |
| 7392 | 60–64 | " " | 1 gm. human skin. (non-irradiated). | Moderate. | Moderate. | | 1.04 |
| 7393 | 60–60 | | | Slight. | Slight. | | |
| 7394 | 70–80 | | | Moderate. | Moderate. | | |
| 7395 | 72–80 | | | " | Slight. | | |
| 7396 | 60–60 | " " | 1 gm. calfskin | No. | No. | | 2.95 |
| 7397 | 74–96 | | *irradiated* ½ hr. | " | " | | |
| 7398 | 60–80 | | at 1 ft. | " | " | | |
| 7399 | 74–90 | | | " | " | | |
| 7400 | 60–64 | " " | 1 gm. calfskin (non-irradiated). | Moderate. | Moderate. | | 1.25 |
| 7401 | 64–68 | | | " | " | | |
| 7402 | 72–84 | | | Marked. | Marked. | | |

skin could be activated after it had been removed from the body. Skin is particularly rich in cholesterol, in fact there is no other organ except the brain which contains a greater total amount of this substance. It seemed therefore probable that the cholesterol

in the epidermis would respond to irradiation. To this end human skin and calfskin were obtained and fed daily in 1 gm. per capita amounts to rats which were receiving a low phosphorus diet. The control series received non-irradiated skin. As will be seen in Table VI the rats which received the irradiated skin were regularly protected from rickets, whereas the control animals developed rickets; the inorganic phosphorus of the blood followed a parallel course. This result is of interest in connection with the recent experiments of Luce (5) who found that "exposure to sunlight appears, however, to have a small effect in determining the antirachitic value of the milk" of the cow.

Cholesterol is an unsaturated sterol having one double bond. It seemed as if the action of the ultra-violet rays may take place at the site of this double bond and therefore that experiments with saturated sterols would be of interest. Accordingly dihydrocholesterol and dihydrophytosterol were irradiated under the above conditions. Both of these preparations were obtained from Dr. R. J. Anderson whom we wish to thank, not only for providing us with them, but also for suggestions in connection with this aspect of the problem. The dihydrophytosterol had been prepared by reducing phytosterol in ethereal solution with hydrogen in the presence of platinum black in the manner described by Anderson and Nabenhauer (6). The dry preparation had the following constants: m. p. 142–143°C.; $[\alpha]_D^{25} = +23.3°$ in chloroform solution. It did not give the Liebermann-Burchard reaction nor did it absorb bromine. The original phytosterol had been isolated from wheat bran (7) and in chloroform solution its specific optical rotation was $-30.9°$.

The dihydrocholesterol had been prepared by reducing the cholesterol in ethereal solution with hydrogen in the presence of platinum black in exactly the same manner as described for dihydrophytosterol. The dihydrocholesterol melted at 142°; and $[\alpha]_D^{23}$ was $+23.45°$ in chloroform solution. It did not give the Liebermann-Burchard reaction and did not absorb bromine. The original cholesterol had been isolated from beef plasma by Professor Bloor of Rochester University. The purified cholesterol showed the following constants: m. p. 142°; $[\alpha]_D^{20} = -39.54°$ in chloroform solution. The melting points given above are uncorrected.

These preparations were irradiated separately, suspended in water, and fed in 0.25 cc. amounts to rats. In contradistinction to the results obtained with the ordinary cholesterol and phytosterol it was found that these saturated preparations failed to be rendered active biologically by irradiation and were unable to

TABLE VII.

*Feeding Experiments with Irradiated and Non-Irradiated Dihydrocholesterol and Dihydrophytosterol.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | |
|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. |
| | *gm.* | | | | | |
| 7354 | 34–38 | Low phosphorus. No. 84. | 0.25 cc. 1 per cent suspension dihydrocholesterol *irradiated* ½ hr. at 1 ft. | Moderate. | Slight. | Moderate. |
| 7355 | 30–38 | | | Slight. | " | " |
| 7403 | 59–66 | | | Marked. | Marked. | |
| 7404 | 60–58 | | | Moderate. | " | |
| 7357 | 50–32 | " " | 0.25 cc. 1 per cent suspension dihydrocholesterol. | Moderate. | Moderate. | Moderate. |
| 7359 | 58–72 | | | Marked. | " | " |
| 7374 | 60–60 | " " | 0.25 cc. 1 per cent watery suspension dihydrophytosterol *irradiated* ½ hr. at 1 ft. | Moderate. | " | " |
| 7375 | 62–70 | | | " | " | " |
| 7376 | 54–60 | | | " | " | " |
| 7377 | 44–50 | | | " | " | " |
| 7378 | 56–68 | Low phosphorus. No. 84. | 0.25 cc. 1 per cent suspension dihydrophytosterol. | " | " | " |
| 7380 | 48–54 | | | " | " | " |
| 7381 | 60–66 | | | " | " | " |

prevent the development of rickets (Table VII). It may be added that spectrograms showed that they had not been altered in their transmission for ultra-violet rays, whereas the transmission of ordinary cholesterol and phytosterol was found to have been changed by irradiation. The details of these spectrographic tests are considered in the paper which follows.

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Two other terpenes were used in the course of this investigation —cymene which has three double bonds in the ring and citronella oil (redistilled) which has one double bond. These unsaturated terpenes were irradiated in the usual way and fed in 0.25 cc.

TABLE VIII.

*Feeding Experiments with Irradiated and Non-Irradiated Cymene and Citronella oil.*

| Rat No. | Weight. | Rickets-producing diet. | Substance fed. | Rickets. | | |
|---|---|---|---|---|---|---|
| | | | | Radiographic. | Macroscopic. | Microscopic. |
| | gm. | | | | | |
| 7360 | 60–60 | Low phosphorus. No. 84. | 0.25 cc. 1 per cent watery suspension cymene *irradiated* ½ hr. at 1 ft. | Slight (20 days). | Slight. | Slight. |
| 7362 | 35–36 | | | Moderate. | Moderate. | Moderate. |
| 7363 | 60–70 | " " | 0.25 cc. 1 per cent watery suspension cymene. | " | " | " |
| 7364 | 36–40 | | | Slight (17 days). | Slight. | " |
| 7365 | 30–34 | | | No (19 days). | No. | Slight. |
| 7423 | 40–44 | " " | 0.25 cc. 1 per cent watery suspension citronella oil *irradiated* ½ hr. at 1 ft. | Moderate. | Moderate. | |
| 7425 | 24–24 | | | " (24 days). | " | |
| 7426 | 40–49 | | | Moderate (28 days). | " | |
| 7427 | 24–34 | " " | 0.25 cc. 1 per cent watery suspension citronella oil. | Moderate. | Marked. | |
| 7428 | 40–40 | | | " | " | |
| 7429 | 30–34 | | | " | " | |

amounts of a 1 per cent aqueous suspension. As may be seen in Table VIII they failed to develop rickets-protective qualities, conferring no protection on the rats which were fed the standard rickets-producing ration.

## CONCLUSIONS.

Dry milk, flour, and spinach can be rendered antirachitic by radiations from the quartz mercury vapor lamp. This potency

was maintained by the spinach after it had been boiled for ½ hour. Oleic acid and egg phosphatide cannot be activated by this means.

Irradiated cholesterol was found to prevent rickets when given by the subcutaneous route.

A series of tests with selective filters showed that the radiations which render cholesterol active biologically are similar in their wave-lengths to those which have been found to protect animals against rickets when they are directly exposed to the rays.

Irradiated cholesterol is effective in preventing the rickets brought about by a diet either low in phosphorus or low in calcium.

Skin which ordinarily possesses no antirachitic potency develops this quality after irradiation with ultra-violet rays.

The saturated reduction products of cholesterol and phytosterol—dihydrocholesterol and dihydrophytosterol—were not activated by irradiation and did not acquire antirachitic properties. The same was true of the unsaturated terpenes, cymene and citronella oil.

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# THE ANTIRACHITIC VALUE OF IRRADIATED CHOLESTEROL AND PHYTOSTEROL.

## III. EVIDENCE OF CHEMICAL CHANGE AS SHOWN BY ABSORPTION SPECTRA.

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(Received for publication, March 28, 1925.)

In previous communications it has been shown that cholesterol and phytosterol do not prevent the occurrence of rickets when fed to rats but that either of these substances acquire this potency as the result of irradiation with ultra-violet rays (1, 2). It was further reported that cholesterol which had been biologically activated by this means protected rats against rickets when given by the subcutaneous route, and also that it was effective whether a diet low in phosphorus or one low in calcium was fed as the basal rickets-producing ration (3). Approximately the same wavelengths of ultra-violet light seem to be involved in this activation as has been shown to be responsible for the protection of rats from rickets when these animals are directly irradiated. The saturated reduction products—dihydrocholesterol and dihydrophytosterol—failed to develop any specific antirachitic activity following irradiation.

Whether we are to consider that a vitamin has been developed in cholesterol by this treatment depends largely on our definition of a vitamin. It is therefore premature and of little value to discuss the question from this point of view. It may be stated, however, that the amount of irradiated substance necessary to bring about protection is exceedingly small and of the magnitude which has been associated with the action of vitamins; less than 1 mg. of irradiated cholesterol suffices to protect a rat which is receiving the low phosphorus Sherman-Pappenheimer diet.



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As previously mentioned the effect of irradiation on cholesterol has been studied by means of spectrograms (1). As is well known the absorption spectrum of a substance is dependent on the orientation of the atoms and electrons composing the molecule as well as on the stoichiometrical constitution, so that under any fixed set of conditions the absorption spectrum of a substance is constant and reproducible. Absorption spectrum measurements may, therefore, be employed as evidence of chemical change.

Absorption measurements have been made, both qualitatively and quantitatively with cholesterol. These tests were carried out by Dr. W. T. Anderson, Jr., of the Hanovia Chemical Company, whose help we wish to acknowledge in this connection. The source of light was the quartz mercury vapor lamp. The qualitative measurements were made with a Hilger quartz spectrograph, the quantitative with a copper-constantan thermopile-galvanometer set. The measurements were made in alcoholic solutions, 0.05 gm. of cholesterol per 25 cc. of alcohol, the layer of liquid traversed by the radiation being 40 mm. The cholesterol was radiated in alcoholic solution, in watery suspension, and dry. The method of radiation appeared to make no material difference in the spectral transmission of the cholesterol nor in the production of the antirachitic factor.

Irradiated cholesterol consistently transmitted more ultra-violet energy than the same suspension or solution which had not been irradiated (Fig. 1). In order to exclude the possible effect of heat, a water-cooled (Kromayer ) lamp was used in the experiment. In various tests of this kind it was found that the irradiated cholesterol transmitted ultra-violet radiations even more intensely than 95 per cent alcohol. The result was the same whether the cholesterol was irradiated in alcoholic solution, in water, or in a dry state. In a test in which spectra were made of cholesterol which had been irradiated for periods of 10, 30, and 60 minutes respectively, it was found that the cholesterol which had been irradiated for the longest period transmitted ultra-violet radiations to the greatest degree, and that an irradiation of 10 minutes duration did not cause a significant spectral change; in all instances the spectrograms were made immediately after irradiation (Fig. 2).

Prolonged irradiation by means of the mercury vapor lamp leads

to an inactivation of cholesterol instead of an activation. Just how long an exposure is necessary to bring about this reversal

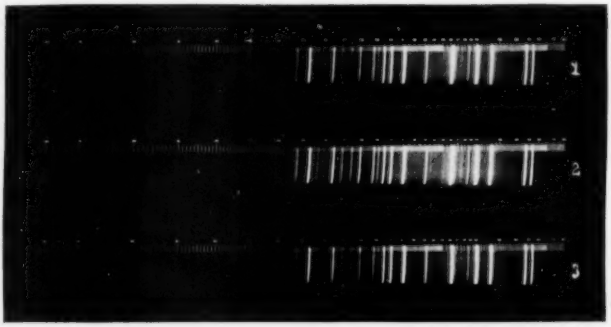


FIG. 1. Irradiated and non-irradiated cholesterol. (1) Mercury vapor lamp (Kromayer); (2) irradiated cholesterol; and (3) non-irradiated.

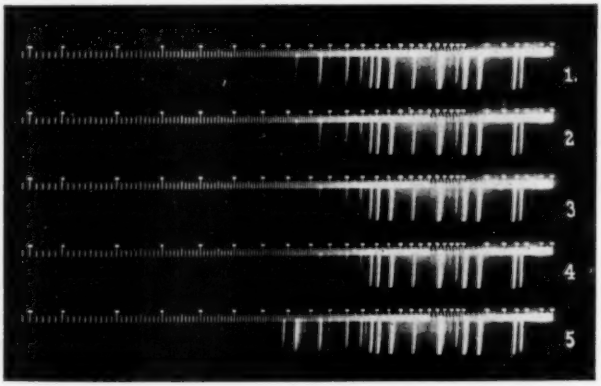


FIG. 2. Irradiated and non-irradiated cholesterol. (1) Irradiated 60 minutes; (2) 30 minutes; (3) 10 minutes; (4) non-irradiated; and (5) alcohol.

has not been ascertained. Cholesterol which had been irradiated continuously for 17 hours not only failed to give evidence of increased transmissibility but showed a lower degree of trans-

missibility than ordinary cholesterol which had not been irradiated (Fig. 3). This was true to a somewhat greater extent in cholesterol which had been irradiated in a watery suspension than in that which had been irradiated in a dry state. Such cholesterol becomes somewhat yellow in color and acquires a lower melting point. In one specimen the melting point was initially 147°C. (uncorrected), which in the course of irradiation fell gradually hour by hour until it reached about 135° after the exposure had been continued for a period of 17 hours. If the cholesterol is irradiated for a still longer time (30 hours or more) it becomes of a decided yellow color and quite opaque to ultra-violet and even to blue radiations. These spectral results conformed with feeding

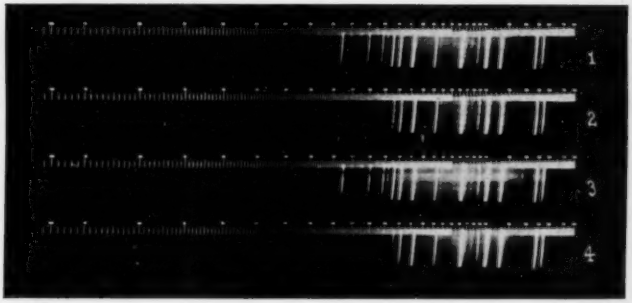


FIG. 3. Effect of prolonged irradiation on cholesterol. (1) Irradiated dry ½ hour; (2) dry 17 hours; (3) in water ½ hour; and (4) in water 17 hours.

tests carried out on animals, which showed that cholesterol irradiated for a long period had lost the specific antirachitic property which it had acquired by irradiation carried out for ½ to 3 hours. Steenbock and his collaborators, who have also demonstrated the power of ultra-violet rays to activate animal and vegetable substances, have reported that vegetable oil loses its potency following prolonged irradiation (4). Apparently cholesterol when subjected to ultra-violet radiations is converted into another substance which is more transparent to these rays. However, the reaction does not cease at this stage, but continues after the exciting radiations have been removed, so that another substance is formed which does not transmit ultra-violet radiations as readily as non-

irradiated cholesterol and does not possess antirachitic properties. This substance possibly may be identical with the yellow compound obtained by the prolonged irradiation of cholesterol.

Various filters were employed in the study of the absorption spectra; window glass, Corning filter G-585—which transmits considerable of the 313 $m\mu$ band as well as 302 $m\mu$ waves of lesser intensity—and a filter of quartz. Ultra-violet rays which had traversed the glass and the Corning filter did not appreciably alter the spectrogram of the cholesterol, whereas those used in association with the quartz filter brought about the expected

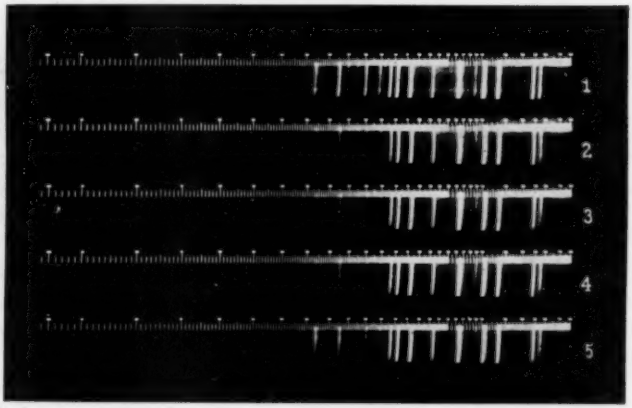


FIG. 4. Irradiation of cholesterol through filters. (1) Quartz; (2) G-585; (3) window glass; (4) not irradiated; and (5) alcohol.

increase in transmission (Fig. 4). It may be added in this connection that experiments with other forms of energy, such as visible light or radiant heat, did not bring about a change in the spectrograms. Nor did irradiation with Roentgen rays affect the cholesterol; soft rays failed to activate it, and soft as well as hard rays failed to alter the activity of the cholesterol which had been irradiated. The Roentgen rays did not seem to have been absorbed by the cholesterol.

The increase in transmission was not due to a production of hydrogen peroxide. In fact, transmission proved to be greater in

tests in which cholesterol was irradiated in alcohol for a period of 3 hours than when 1 cc. of 0.001 or 0.00001 normal hydrogen peroxide solution had been added. In these tests, as in some others, the irradiated cholesterol transmitted the light rays even better than the 95 per cent alcohol.

As dihydrocholesterol and dihydrophytosterol had failed to be activated biologically by irradiation, as demonstrated in feeding experiments, these substances were tested from the point of view of spectral absorption. Spectrograms showed that they are but little altered in this respect by subjection to ultra-violet irradiation; what little change was produced seemed to be in the direction of a diminished transmissibility (Fig. 5).

As we proceeded with these tests it became evident that cholesterol which has been changed in its spectral absorption by irradiation does not maintain this state. For example, if a second spectrogram is made 1 to 2 weeks later it is found that the spectrum has reverted toward its original form and that the cholesterol no longer has its heightened degree of transmissibility (Fig. 6). This deterioration or "ageing" takes place whether the cholesterol has been irradiated originally in the dry state, suspended in water, or dissolved in alcohol. The spectrogram may show not only no increase in transmission but be even inferior in this respect to cholesterol previous to irradiation. In other words, we have brought about a decrease rather than an increase by irradiation. This aspect was shown more strikingly by the use of filters. The cholesterol which had been irradiated through window glass and failed of activation was found to have a higher transmission after standing for 2 weeks than the cholesterol which had been simultaneously irradiated through quartz and which at the outset had been activated (Fig. 7).

As has been stated, quantitative measurements were carried out by means of a galvanometer set. The results of some of these tests are portrayed in the accompanying graph (Fig. 8). It may be noted that there is a gradual increase in the deflection of the galvanometer with the irradiated cholesterol, that this increase is followed by an even plane and is succeeded later by a decrease after irradiation has been carried out for a prolonged period. Another curve shows the effect of "ageing" and illustrates that this is a gradual and continuous process, steadily maintained for a

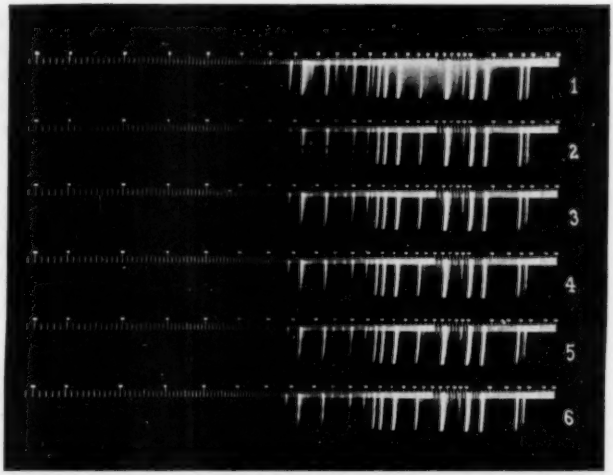


FIG. 5. Irradiation of dihydrocholesterol and dihydrophytosterol. (1) Alcohol; (2) cholesterol not irradiated; (3) dihydrocholesterol irradiated 20 minutes; (4) dihydrocholesterol not irradiated; (5) dihydrophytosterol irradiated 20 minutes; and (6) dihydrophytosterol not irradiated.

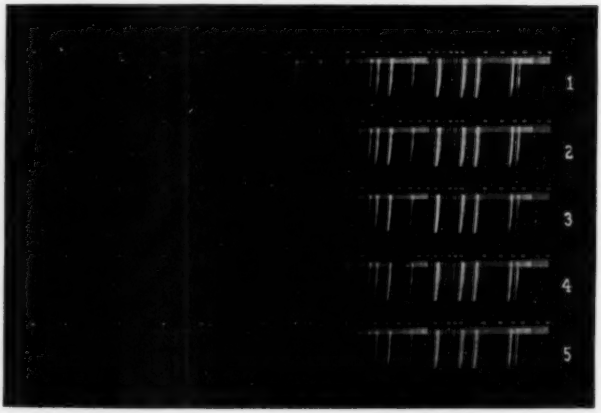


FIG. 6. Effect of "ageing" on irradiated cholesterol. (1) Alcohol; (2) non-irradiated cholesterol; (3) irradiated dry ½ hour and aged; (4) irradiated in water ½ hour and aged; and (5) irradiated in alcohol ½ hour and aged.

period of many weeks. It may be added that the cholesterol which is charted as recording a deflection of 130 mm. immediately

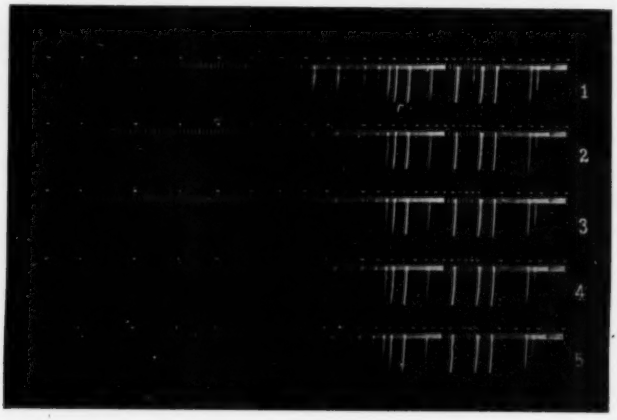


FIG. 7. Effect of "ageing" on cholesterol irradiated through filters. (1) Alcohol; (2) not irradiated; (3) through quartz and aged; (4) G-586-A and aged; and (5) window glass and aged.

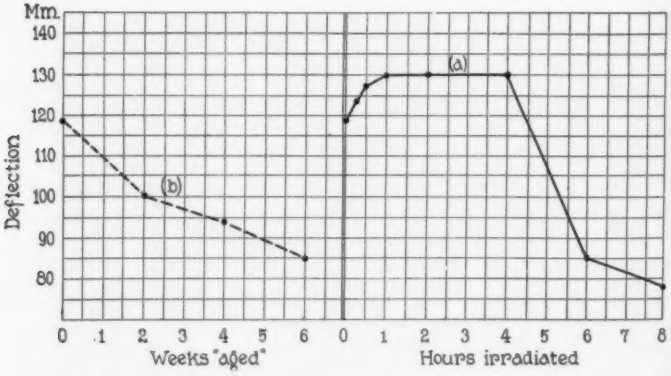


FIG. 8. Galvanometer deflections (*a*) of cholesterol irradiated in alcohol or dry for various periods and (*b*) of activated cholesterol which had "aged" for a succession of weeks after having been irradiated for ½ hour.

after irradiation for 1 hour, gave a reading of only 100 mm. when tested 1 week later.

This spontaneous change in spectral absorption calls to mind the variation in potency which takes place in the active principle of cod liver oil prepared by Zucker's method. This preparation although stable when dissolved in oil becomes rapidly inert when kept in a dry state. So in the case of the antirachitic principle elaborated by means of irradiation. The activated cholesterol becomes inactive, as judged by animal experiment or by spectral absorption, when kept in water or in a dry state, but retains its potency longer in a menstruum of oil. As previously shown irradiated cottonseed oil retains its protective properties for at least 6 months.

## SUMMARY.

By means of spectral absorption tests a chemical change was demonstrated in cholesterol which had been endowed with antirachitic potency by ultra-violet irradiation. This activated cholesterol absorbs ultra-violet radiations to a less degree than does ordinary cholesterol, an effect which is intensified with increasing degrees of irradiation. If, however, irradiation is prolonged for many hours the activated cholesterol becomes less transparent than even non-irradiated cholesterol. This comparatively opaque product is of a yellowish color and has a lowered melting point. Visible light, radiant heat, and Roentgen rays do not alter the absorption spectrum of cholesterol. Neither dihydrocholesterol nor dihydrophytosterol undergoes a spectral change as the result of irradiation. When activated cholesterol is kept in a watery suspension or in a dry state its spectral transmission becomes gradually diminished until it reaches a point where it transmits less than it did originally.

These spectrographic results were tested and confirmed quantitatively by means of a thermopile-galvanometer set.

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# MODIFICATION OF BLOOR'S METHOD FOR THE DETERMINATION OF CHOLESTEROL IN WHOLE BLOOD OR BLOOD SERUM.

By GUY E. SACKETT.

*(From the Laboratory of Drs. Myers, Kansas City.)*

(Received for publication, February 26, 1925.)

Although Bloor's method[1] for the determination of cholesterol in blood has been considered a standard method and has been widely used, it has several disadvantages which have prevented it from being universally accepted. Some of these are the amount of reagents, blood, and time required to make the test, and also the fact that so frequently a brown color develops in the unknown which makes it hard to read accurately in the colorimeter against the clear green standard.

The following modification has been developed by the author with the intention of overcoming some of these disadvantages.

The protein is precipitated with a mixture of alcohol and ether and the cholesterol in the filtrate is estimated colorimetrically against a standard solution of cholesterol.

## *Method.*

Put 9 cc. of alcohol in a 15 cc. graduated centrifuge tube, add 3 cc. of ether, and mix by inverting. Run in slowly 0.2 cc. of whole blood or plasma. Cork tightly and shake vigorously for about 1 minute. Let lie horizontally with the sediment evenly distributed along the tube for 30 minutes. Centrifuge rapidly for 3 minutes and decant into a small beaker. Evaporate just to dryness in a water bath. Extract the cholesterol twice for about 2 minutes each time with small portions (2 to 2.5 cc.) of chloroform and decant into a 10 cc. glass-stoppered graduated cylinder. Let cool and then make up to 5 cc.

Measure 5 cc. of a standard cholesterol solution in chloroform (containing 0.4 mg. of cholesterol) into a similar 10 cc. cylinder.

[1] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.



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To each of the solutions add 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid. Mix by inverting several times and then set away in the dark for 15 minutes. Transfer to the cups of the colorimeter and compare as usual, setting the standard at 12 or 15 mm.

$$\textit{Calculations.}\quad \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 200 = \text{mg. per 100 cc. of blood}$$

It is convenient to make the cholesterol standard in two strengths: (*a*) the stock solution containing 0.2 gm. of cholesterol in 200 cc. of chloroform; and (*b*) the standard solution for use made by diluting 8 cc. of the above to 100 cc. with chloroform. 5 cc. of this latter solution will contain 0.4 mg.

This modification appears to have the following advantages over Bloor's method: (1) less alcohol and ether required; (2) the brown color, which develops in the unknown, eliminated; (3) less time required for the determination; and (4) less blood required for the determination.

*Cholesterol Content of Whole Blood.*

| Name. | Bloor's method. | Author's modification. |
|---|---|---|
| | *mg. per 100 cc.* | *mg. per 100 cc.* |
| P. B. (3 pooled bloods) | 218 | 216 |
| A. N. (Miss) | 220 | 221 |
| D. S. W. (Mrs.) | 288 | 280 |
| S. M. (Mrs.) | 277 | 263 |
| E. T. M. (Mr.) | 182 | 193 |
| J. L. M. (Mr.) | 205 | 212 |
| O. B. (Mrs.) | 235 | 238 |
| J. A. J. (Mr.) | 186 | 184 |
| F. H. (Mr.) | 169 | 176 |
| L. A. W. (Mrs.) | 274 | 260 |
| L. V. S. (Mr.) | 195 | 184 |
| O. (Mr.) | 117 | 115 |
| R. A. (Miss) | 208 | 204 |
| G. J. (Mr.) | 168 | 183 |
| P. H. (Mrs.) | 203 | 214 |
| P. (Mrs.) | 216 | 207 |
| M. K. (Miss) | 238 | 222 |
| Average | 211.7 | 210.1 |

A known amount of cholesterol was added to blood, the cholesterol content of which was known, and was recovered as follows:

| Blood with cholesterol content + amount of cholesterol added. | Calculated. | Author's modification. |
|---|---|---|
| *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* |
| A. N. (220) + 160 | 380 | 362 |
| J. A. J. (185) + 200 | 385 | 386 |
| M. K. (230) + 200 | 430 | 422 |

# THE DETERMINATION OF BLOOD SUGAR.

By STANLEY R. BENEDICT.

*(From the Department of Chemistry, Cornell University Medical College, New York City.)*

(Received for publication, March 26, 1925.)

There are at present available several methods for the determination of sugar in blood which yield satisfactory results for following the blood sugar in diabetes. These include the picric acid procedure in one or other of its various modifications and the copper methods of Bang, Shaffer and Hartman, and Folin and Wu. To many it may appear unjustifiable to include the picric acid methods here, but the writer ventures to assert that although many thousands of analyses have been made by the picric acid methods, it is doubtful whether a clinician has ever been misled in his interpretation of a diabetic case by the figures obtained for these analyses when properly carried out. Nevertheless, critical studies of the picric acid methods have shown that these consistently yield figures higher than the actual glucose content of the blood. This does not mean, however, that we should not regard the use of the picric acid methods as a legitimate routine procedure by the clinician. Indeed we have frequently noted a fact which indicates that the picrate method may be preferable to a copper method for following the course of the blood sugar in the diabetic. With diet, the blood sugar of the diabetic tends to return more promptly to the normal level as indicated by a copper method (Folin-Wu) than when the determination is made with the picrate method. This point is worthy of further study and we are accumulating data along this line as opportunity permits. We would point out in this connection that for bloods having a normal non-protein nitrogen content the picrate method yields figures practically identical with those given by the Folin-Wu procedure, *if applied to a portion of the same tungstic acid filtrate* after concentrating a portion of the filtrate by boiling so that the sugar



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concentration is high enough to permit an accurate determination by the picrate procedure. Though Csonka and Taggart[1] report results somewhat at variance with this statement it must be noted that their figures show a much closer agreement when both methods were applied to the tungstic acid filtrate, and that Csonka and Taggart apparently took no precautions to insure a proper concentration of sugar for the picrate reaction. We have carried out comparative determinations upon some seventy (70) bloods, chiefly human, and have found in nearly every instance a close agreement by the two methods when applied to the tungstic acid filtrate, providing there was no appreciable nitrogen retention in the blood. The point is of interest as showing that the difference by the two methods for normal blood is due to some unknown non-protein constituent, which is precipitated by tungstic acid and not by the acid picrate solution. The point deserves further investigation.

With the discovery of insulin it has become a matter of considerable importance for a true understanding of sugar metabolism in the body to be able to measure the actual glucose content of the blood just as closely as may be possible. It seems very probable to the present writer that one or more of the non-glucose-reducing bodies of the blood is directly concerned in sugar metabolism. Obviously this point can be studied adequately only as we may be able more and more sharply to limit at least one method strictly to the glucose in the blood. It is doubtful whether this end is yet in sight, but it is the purpose of the present paper to present a new method for blood sugar determinations, which employs a more specific copper reagent than has hitherto been available, and which yields figures for the blood sugar very appreciably lower than those heretofore obtained.

The greater delicacy and specificity of copper reagents containing carbonate in place of hydroxide were pointed out by the present writer many years ago.[2] In that paper a reagent was proposed for sugar detection which contained copper sulfate, Rochelle salt, and sodium carbonate. This reagent is more delicate than the citrate reagent subsequently proposed, but was not

[1] Csonka, F. A., and Taggart, G. C., *J. Biol. Chem.*, 1922, liv, 1.
[2] Benedict, S. R., *J. Biol. Chem.*, 1907, iii, 101.

so stable nor so satisfactory, because of its great sensitiveness as a general qualitative reagent for sugar in urine. Folin and Wu, in constructing their admirable method for blood sugar determination, adopted the tartrate carbonate copper reagent in modified form. The chief point of modification was in dilution. Folin and Wu thus obtained an exceedingly sensitive reagent. It is, however, a fact, as the writer learned when first studying the carbonate reagents, that dilution of such reagents increases the sensitiveness at the cost of some of the specificity. Apparently the copper complex is more ionized in the greater dilution. Thus the Folin-Wu modified copper solution is readily reduced by creatinine, formaldehyde, creatine, chloroform, etc. We do not believe that creatine, creatinine, or other *known* non-glucose-reducing bodies occur in the blood in large enough amount to affect appreciably this reagent; yet we have felt for a long time that a forward step in blood sugar analysis would be taken if a copper solution could be found which would be satisfactorily sensitive for blood sugar determination, and at the same time which would not be appreciably reduced by such known non-sugar-reducing compounds as may occur in blood or urine.

If the Folin-Wu copper reagent is applied directly to normal urine it will show from two to three times as much sugar as is present. Obviously it is theoretically not wholly satisfactory to apply such a solution directly to blood filtrates and assume that all the reduction obtained is due to glucose. We have no wish in this connection to stress any criticism of the procedures elaborated by Folin and by Shaffer for determination of blood sugar. We admire these methods and have employed them freely for a long time. We merely mention the fact to explain why we have kept in mind developing a solution which should give approximately the sugar content of normal urine directly, so that we could find out whether the application of such a solution to blood filtrates would result in lower figures for the blood sugar.

More than 2 years ago we devised a copper solution which could be applied directly to urine with an error of about one-tenth that obtained with the Folin-Wu solution. Indeed in many samples of urine this reagent yielded as low figures for sugar when applied directly as did the Folin-Wu solution after treatment of the urine with Lloyd's reagent. There were, however, difficulties in the

application of the solution to the blood because the quantity of reduced copper oxide yielded by a given amount of glucose was not great enough, and because of difficulty in finding a suitable reagent for color development after the reduced copper oxide was obtained. About a year ago, however, we solved these difficulties, and have since that time employed the method under widely varying conditions so as to test its accuracy as thoroughly as possible.

The new developments in reagents and technique are essentially the following. The copper reagent employed is a modification of the qualitative citrate carbonate solution, in which the concentration of citrate has been increased very materially, while the quantity of copper and carbonate has been reduced. A small amount of sodium bisulfite has been added to the reagent, which results in a remarkable increase in the quantity of cuprous oxide obtained from the small amount of sugar contained in the dilute blood filtrate. The addition of sulfite in this connection is apparently a new departure in sugar analysis. Its action is probably through combining with fragments of the sugar molecule and preventing their destruction by the carbonate present until they have reduced the copper. Certainly the action is not due to the sulfite preventing the reduced copper from being reoxidized, because carrying out the reaction in the complete absence of atmosphere oxygen will not result in the increased yield of cuprous oxide. The sulfite itself has no reducing action upon the copper solution.

For the development of color by cuprous oxide we make use of the tungstic-arsenic-phosphoric acid reagent which we proposed sometime ago for uric acid determination,[3] and to which is added about 5 per cent of commercial formalin. Unless the formalin is added the sulfite present in the copper reagent will produce some color. The color reagent of Folin and Wu cannot be used because the color obtained in the presence of the new copper reagent is very weak and fades almost immediately.

Our final solutions have slightly more color from the same amount of sugar than is obtained in the Folin-Wu method, and the color shows little tendency to change on standing.

[3] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.

In order to make for simplicity and uniformity we have made our reagents and technique so that the new process is nearly identical in quantity of reagents used and general technique with that of Folin and Wu. The exact procedure for the method follows.

*Reagents.*

*1. Alkaline Copper Solution.*—

| | |
|---|---|
| Pure crystallized copper sulfate | 6.5 gm. |
| Sodium citrate | 200 " |
| " carbonate (anhydrous) | 50 " |
| " bisulfite | 1 " |
| Distilled water to make | 1,000 cc. |

Dissolve the citrate, carbonate, and bisulfite together in about 850 cc. of water. Dissolve the copper sulfate separately in about 100 cc. of water and add to the other solution, with constant stirring or shaking. Dilute the solution to 1 liter. This solution will keep for a year or longer.

*2. Complex Tungstic Acid Color Reagent.*—This is prepared as follows: 100 gm. of pure sodium tungstate are placed in a liter flask and dissolved in about 600 cc. of water. 50 gm. of pure arsenic pentoxide are now added, followed by 25 cc. of 85 per cent phosphoric acid and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes. After cooling, 50 cc. of commercial formalin are added and the solution is diluted to 1 liter.[4]

The technique of the method is as follows: 2 cc. of the 1:10 tungstic acid filtrate are measured into a Folin-Wu sugar tube,[5] followed by 2 cc. of the copper reagent. The contents are mixed by side to side shaking for a moment and placed in boiling water for 4 to 5 minutes. The tubes are then cooled by immersion in cold water and 2 cc. of the tungstic-arsenic acid reagent are added. At the end of 6 to 10 minutes the contents of the tubes

[4] Except for the formalin, this reagent is identical with that proposed by the writer for determination of uric acid in blood. If desired, 5 cc. of formalin can be added to 100 cc. of the uric acid reagent previously prepared, and this solution used for color development in the sugar method.

[5] In place of the special Folin-Wu sugar tube an ordinary test-tube graduated at 12.5 and 25 cc. may be used with the following simple precaution to prevent reoxidation of the cuprous oxide. After the solutions are placed in the tube 2 or 3 drops of benzene are added and the tube is plugged with cotton. During the heating the benzene is completely vaporized. The heavy vapor remains in the tube and excludes the air. In the presence of the benzene apparently much more cuprous oxide is precipitated. This is due to the oxide being in a finer state of division. The final results are the same as where the Folin-Wu tube is used.

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are diluted to 25 cc. with water and compared with a standard in a colorimeter. As a standard solution, pure glucose solution containing 0.1 and 0.2 mg. of glucose per cc. is employed just as in the Folin-Wu process. These can be conveniently prepared by proper dilution of a 0.1 per cent aqueous solution of glucose, to every liter of which a few drops of toluene have been added. Such a solution will keep unchanged for years. The diluted standards will also keep for long periods in the presence of a few drops of toluene.

In Table I the results of comparative analyses by the new method and by the Folin-Wu procedure are recorded for fourteen

TABLE I.

*Showing Comparative Figures for Blood Sugar by the Folin-Wu and by the New Procedure.*

| Blood No. | Source. | Folin-Wu method. | New method. | Results by Folin-Wu method / Results by new method |
|---|---|---|---|---|
| | | *mg. per 100 cc.* | *mg. per 100 cc.* | |
| 1 | Human. | 73 | 65 | 1.12 |
| 2 | " | 89 | 73 | 1.21 |
| 3 | " | 100 | 84 | 1.19 |
| 4 | " | 95 | 76 | 1.25 |
| 5 | " | 100 | 79 | 1.26 |
| 6 | " | 73 | 61 | 1.19 |
| 7 | " | 88 | 73 | 1.20 |
| 8 | Dog.* | 128 | 107 | 1.19 |
| 9 | Human (mild diabetes). | 152 | 120 | 1.26 |
| 10 | " (diabetes). | 320 | 277 | 1.15 |
| 11 | " ( " ). | 296 | 246 | 1.20 |
| 12 | " ( " ). | 516 | 448 | 1.12 |
| 13 | " ( " ). | 333 | 304 | 1.11 |
| 14 | Dog (in insulin convulsions). | 27 | 16 | 1.68 |

* 20 hours after ligation of both ureters.

blood samples. With a few obvious exceptions, these figures are taken at random from the large number of comparative analyses which we have made. It will be noted that the new method yields figures averaging some 20 per cent lower than those by the Folin-Wu procedure. The discrepancy between some of the figures is so great as to lead to the suspicion that the new method fails to record all the glucose present in the blood. In this connection, we would point out that glucose added to blood filtrates is recovered satisfactorily by the new procedure. The different results

by the new method become more comprehensible when we bear in mind that if the two methods be applied directly to diluted normal urine the new procedure consistently yields figures about one-third as great as those given by the Folin-Wu modified tartrate reagent. It is hardly surprising that the blood should show differences of the same kind, though of very much less magnitude.

The figures reported for the sugar of Sample 14 merit a word of comment. The dog from which this sample of blood was obtained was in convulsions after an enormous dose of insulin. Figures by both the Folin-Wu and the Shaffer-Hartman methods showed a sugar content for the blood of 27 mg. per 100 cc. The new procedure reduces this figure to 16, a difference of 68 per cent. As a matter of fact, we are inclined to regard this blood as free from glucose, for we believe that the new method still yields results averaging perhaps 15 mg. per 100 cc. too high. Our chief reason for this view is that all the copper methods which we have so far tested will give a definite increase in the figure for sugar if the heating of both standard and unknown is continued beyond the period required for complete oxidation of the sugar in the standard tube. In the Folin-Wu method the increase in sugar is as much as 15 to 20 mg. per 100 cc. of blood if the heating is continued 12 to 18 minutes instead of the 6 minutes directed. The new method shows the same curve of increase though quantitatively less. Unless we assume an inhibiting substance in the blood (which cannot be demonstrated and which would invalidate all copper methods as applied to blood) we must assume the presence of a slowly reacting interfering compound which causes plus errors of something over 10 mg. per 100 cc. of blood.

Figures by the new method indicate that the normal blood sugar content averages about 75 mg. per 100 cc. We are, however, of the opinion that the actual glucose content of normal human blood does not usually exceed 60 mg. per 100 cc. except for a period after food ingestion. The figure 60 may still be too high.

It is obvious that the new method can be readily substituted in the various modifications of the Folin-Wu procedure which have appeared, providing for the use of less blood, and which represent essentially a division though by 10 of the quantity of blood and reagents employed.

In a later paper we shall discuss the application of the new copper reagent to the determination of sugar in normal urine.

XUM

# THE DETERMINATION OF URIC ACID IN THE BLOOD.

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(Received for publication, March 26, 1925.)

In a recent number of this Journal, Bulmer, Eagles, and Hunter[1] have published a study of the direct determination of uric acid in the blood as proposed by the present writer,[2] in which the conclusion is reached that figures obtained by the direct method are "unreliable."

We feel that a few words of comment upon that paper may be of service in connection with the still unsettled question of the determination of uric acid in the blood.

The larger proportion of the experimental work recorded by Bulmer, Eagles, and Hunter is concerned with analyses of animal blood of various species (rabbit, cat, dog, guinea pig, and ox). The investigators feel that, if the method cannot be applied to animal bloods and yield correct results, this constitutes presumptive evidence against its use in human blood. Such a conclusion hardly seems warranted. The present writer was fully aware that the direct method could not be applied to animal bloods, and so stated at the time the method was described. If methods were to be rejected because of what might be present in blood or urine, it is doubtful whether any of our present analytical methods for these fluids would survive. Fortunately the position taken by Bulmer, Eagles, and Hunter is not generally accepted, as is evidenced by the continued use of Folin's standard method for creatinine determination in urine in spite of the fact that urine of diabetics frequently contains enough acetone and diacetic acid to render the method inapplicable. Indeed, Bulmer, Eagles, and

[1] Bulmer, F. M. R., Eagles, B. A., and Hunter, G., *J. Biol. Chem.*, 1925, lxiii, 17.

[2] Benedict, S. R., *J. Biol. Chem.*, 1922, li, 187.



XUM

Hunter would, according to their line of reasoning, have to abandon uric acid determination altogether, for as these writers seem to recognize, the figures they report by the precipitation method for animal bloods are so high as to have really little relationship to the true uric acid content of these bloods, with the exception of ox blood. It is regrettable that such highly inaccurate figures should get into the literature under any guise. It would have seemed preferable to state merely that the direct method yields results which are wholly inaccurate for animal bloods—a fact which the present writer would certainly not question. The argument is hardly strengthened by showing that the authors were unable to determine uric acid in animal bloods with reasonable accuracy by any method.

In connection with their analyses of human bloods Bulmer, Eagles, and Hunter have clearly established the interesting fact that in one condition, *viz.*, nickel rash, which may be perhaps classed as at least moderately rare, the direct method for uric acid in blood may give figures definitely too high. This fact seems to us to represent the only argument adduced against the direct method which should have the slightest weight. The evidence here would have been more clear-cut had the writers checked their indirect results by the alkaline silver magnesia precipitation—a procedure more specific for uric acid than any other available. Even accepting that in nickel rash the direct method yields results too high, it would still seem a question in the present writer's mind as to whether such a finding should offset the fact that in hundreds of routine analyses by the direct method only a few scattered cases occur where there is a significant difference by the two methods. Indeed, Table V of Bulmer, Eagles, and Hunter would perhaps raise the question in the minds of many readers as to whether these writers did not go too far in the sweeping conclusion which they reached. In the study reported in Table V, Bulmer, Eagles, and Hunter especially wished to include cases showing wide divergence by the two methods, yet in spite of the fact that these investigators apparently had access to "nickel rash" cases and apparently did their best to find divergent bloods from any source, only two out of fourteen bloods analyzed by the two methods show a difference as great as 1 mg. of uric acid per 100 cc. of blood. Indeed the extensive row

of almost perfect duplicates offered in that table seems rather impressive and in favor of the direct method.

It is therefore at least still an open question as to whether the direct method for uric acid should be abandoned. Against this method we find the fact that nickel rash cases apparently yield high results. There is the further fact that of the usual run of human bloods a certain small proportion—perhaps 5 to 10 per cent—will show figures appreciably higher by the direct method. In favor of the use of the method we have its greater simplicity and therefore in the hands of most analysts, its greater safety as a routine procedure.

The opinion of the present writer is as follows: All analytical methods have some limitations and should be used with due regard to these limitations. The direct method for uric acid should, we believe, be first choice at present as a routine procedure in the analysis of human bloods. When special cases or groups of cases are being studied frequent checking by the precipitation method should be employed, and if a divergence is found the precipitation method should be adopted for such bloods. Any unusual or peculiar condition (nickel rash, for instance) or any case showing high uric acid where there is no other determinable retention or kidney involvement would at once call for use of a precipitation method. Where such simple rules are kept in mind the use of the direct method is wholly justifiable and will save a great amount of time as well as expense for silver, and avoid errors due to incorrect technique. The writer clearly recognized these facts and proposed two precipitation methods for use as checks, the more convenient one of which was adopted throughout their work by Bulmer, Eagles, and Hunter.

We propose now to discuss a few points raised by Bulmer, Eagles, and Hunter in connection with the substance responsible for interference in the direct method. We shall also attempt to correct certain errors which are creeping into the literature of uric acid determination, and which find a place in the discussion of Bulmer, Eagles, and Hunter.

In their paper, Bulmer, Eagles, and Hunter[3] state concerning the probable interfering substance: "(Substance X) . . . . it is not precipitated by silver lactate." The incorrectness of this

[3] Bulmer, Eagles, and Hunter,[1] p. 22.

statement is clearly shown in our paper on direct determination of uric acid in blood. We discussed this point in detail and introduced a special table which showed that the interfering substance *is* precipitated by silver lactate and that the fact that it does not appear in the final uric acid fraction in the Folin-Wu method is because the silver compound is not appreciably decomposed by the acid sodium chloride mixture employed. The idea repeatedly championed by Bulmer, Eagles, and Hunter that silver lactate is a highly specific reagent for the precipitation of uric acid is without the slightest foundation in fact. Acid silver lactate is not to be classed in any way with ammoniacal silver magnesia mixture as a specific precipitant for uric acid. Folin and Wu adopted silver lactate as a uric acid precipitant not because it was more specific than the ammoniacal solution, but because it would precipitate uric acid from the highly dilute blood filtrates which they wished to use. That Folin and Wu clearly recognized the lessened specificity of the acid silver lactate is shown by their introducing the extra step of decomposing the precipitate with acid sodium chloride solution, whereas accurate results could be obtained with the silver magnesia mixture when the entire precipitate is dissolved in cyanide. It should be clearly carried in mind that when dealing with any unknown mixtures, especially fluids from lower animal forms, or bacteria, etc., the test for the qualitative or quantitative presence of uric acid should involve preliminary precipitation with ammoniacal silver magnesia reagent. As the final criterion of the accuracy of a uric acid method preliminary precipitate with ammoniacal silver magnesia mixture stands first, providing a high enough concentration of uric acid (0.2 mg. of uric acid in not over 5 cc.) is used. Such statements as the following by Bulmer, Eagles, and Hunter—"We thus suspect that at least some of substance X is precipitated by the old alkaline uric acid precipitant"—are wholly misleading and are without the slightest experimental justification from any angle. Silver in weak acid solution has hundreds or thousands of known insoluble compounds. In the presence of an excess of ammonia on the contrary, iodide and the purines are practically the only known commonly occurring compounds which form insoluble derivatives with silver. In their paper Bulmer, Eagles, and Hunter state[4] that "The contents

[4] Bulmer, Eagles, and Hunter,[1] pp. 31–32.

of Table V . . . . provide indications of the presence of a hitherto unrecognized substance in human blood. This substance is characterised by the facts that: (*a*) it is not precipitated along with uric acid from protein-free blood filtrates by silver lactate, (*b*) it is for the most part found in the corpuscles of human blood, and (*c*) it practically disappears on standing. It is, further, not 'combined uric acid.'" Had these writers read our discussion of the direct determination of uric acid in blood and considered the findings presented in Table II of that paper,[5] we feel that they could hardly have made such a reference to an "unsuspected" substance. We clearly showed that human blood contains one or more substances other than uric acid which give the uric acid reaction by the carbonate-phosphotungstic acid method, and which are precipitated by silver lactate. We further showed that the silver compound is not decomposed by acid sodium chloride solution, but that if the silver precipitate is dissolved directly in cyanide, results are very much too high by the old process. We had also found, though we did not specifically so state, that precipitation with silver and dissolving in cyanide did not alter the results by the direct method from those obtained when this method is applied directly to the blood filtrate. It is obvious that had such results been lower we could not have advocated the use of the new method directly on the blood filtrate.

So impressed were we with the presence of the uric acid-reacting compound precipitated by the silver lactate that nearly 2 years ago a research was initiated upon the isolation of the compound in question. The work was difficult and progress slow. Nevertheless, within the last few months we have isolated over a gm. of the compound in pure crystalline form from the blood of one species, and have proved the presence of the substance (in surprisingly large quantity) in the blood of every species (including human) so far examined.

We are now engaged in a study of the composition and properties of the new compound, and will report concerning it as promptly as possible. A simple quantitative method for its determination in blood, tissues, and urine has been devised and we are by this means studying its distribution and variation in health and disease.

[5] Benedict,[2] pp. 200-203.

XUM

# A SIMPLE METHOD FOR OBTAINING CUTANEOUS (CAPILLARY) BLOOD FROM INFANTS AND ADULTS FOR COLORIMETRIC pH DETERMINATION.

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(Received for publication, March 6, 1925.)

## INTRODUCTION.

The technique reported below furnishes a simple and convenient method for obtaining blood from infants for studies of the acid-base balance by means of pH and $CO_2$ determinations. It is also suitable for repeated tests on adults.

The technique is described below for the pH determinations.

The total $CO_2$ content of the whole blood, serum, or plasma can be determined by using Van Slyke and Neill's technique on from 0.2 to 1 cc. of sample, or the bicarbonate concentration may be determined on 1 cc. of serum or plasma by the Van Slyke titration method.

The method can also be used in other studies where change in gaseous content is to be avoided, as in the determination of the oxygen content.

The direct determination of the pH of blood plasma by Cullen's colorimetric method has proven a convenient and accurate method for studying conditions of abnormal acid-base balance.

In this method, 0.2 to 1 cc. of the plasma, obtained without stasis or loss of $CO_2$ during manipulation, is added to 20 volumes of 0.9 per cent NaCl solution containing phenol red indicator. The pH readings of this diluted plasma are corrected to the actual pH of the plasma at body temperature by a constant which includes corrections for dilution, temperature, and protein error, and which has been determined by comparison of electrometric and colorimetric readings.



XUM

Hitherto such studies have been carried out on blood obtained from human arteries or veins. Most of the studies made on infants have been made on venous blood obtained through the fontanel. This method may be dangerous in unskilled hands.

Hawkins, working with small animals, rats, mice, etc., found it possible to economize on blood by diluting the blood directly before centrifuging. After centrifuging, the dye color in the diluted plasma could be read without further transfer. This is plasma, since fibrin does not precipitate when blood is diluted with 20 volumes of 0.9 per cent NaCl solution. It has been possible to use this technique with capillary blood of both infants and adults.

*Collection of Blood.*

*Infants.*—The technique consists of adding the necessary precautions to avoid loss of $CO_2$ to the method, common in Denmark, for obtaining blood from children.

The technique for blood pH determination follows. The entire foot is placed in hot water (45-50°C.) until a good hyperemia appears and is then dried with ether. Then the heel is dipped into an ordinary glass funnel of about 55 mm. diameter which is closed at the bottom of the V and which is filled with paraffin oil. A helper holds the funnel with one hand and the infant's leg with the other. First, one grasps the front of the foot firmly with the left hand and with the right brings a small very sharp knife under the oil surface and makes a deep stab incision on the under side of the heel, somewhat to the side of the middle; one then holds the funnel oneself and makes sure that the cut remains under the oil while the blood drops through it. When enough blood has collected for the tests required, from 0.5 to 4 cc., the funnel is removed. At once with a 1 cc. pipette graduated to 0.01 cc. one draws up first 0.20 or 0.3 cc. of oil, then blood, and then transfers the 0.4 cc. of blood to the one comparison tube containing 5.0 cc. of 0.9 per cent NaCl solution and phenol red, overlaid with oil, and another 0.4 cc. to the same amount of 0.9 per cent NaCl solution without indicator. Each tube is then stoppered with a one-hole stopper so that all the oil is expressed, and the hole is closed with a glass plug. The tubes are then centrifuged for 2 to 5 minutes and are ready for comparison with standard solution.

Assuming in infants 62 per cent plasma (Drucker (1923, *a*)), 0.4 cc. of blood is equivalent to 0.25 cc. of plasma. This added to 5 cc. of 0.9 per cent NaCl solution gives the 21 × dilution required. As a matter of fact, the error introduced by using 0.25 or 0.30 cc. is negligible, providing the proper amount of indicator is used.

*Adults.*—An ear is first cleaned energetically with ether, dried, and dipped into a funnel containing paraffin oil. An incision is made under the oil surface on the under side of the lobe in exactly the same manner as with the child's heel. A finger may be used if desired.

*Colorimetric Reading.*—The pH of the diluted plasma is then read in a small Walpole comparator against Sörensen's standard phosphate solutions prepared at 0.05 pH intervals. One easily reads to within 0.01 or 0.02 pH. The cells in the bottom do not interfere with the reading, but the reading must be made within ½ hour.

The temperature of the diluted plasma is read. The pH of the blood at 38° is then calculated, $pH_{38°} = pH_{color\ \cdot t°} + 0.01\ (t - 20°) - 0.26$.

The 0.26 is Cullen's constant for human plasma plus the correction for the loss of $CO_2$ in the oil of 0.03 to 0.04 pH, see below. The 0.01 $(t - 20°)$ is the correction for change in room temperature.

The average of the original determinations lay between 0.22 and 0.23. Hitherto 0.22 has been used although the average was nearer 0.23 than 0.22. Since 0.23 is the more exact value, it will be used here.

### *Details of Technique.*

*The knife*, a cataract knife of about 2 × 30 mm., must be razor-sharp (see Drucker (1923, *b*)). The incision is usually about 5 mm. deep and perhaps 2 mm. wide.

*The tubes* are clear glass tubes 13 × 54 mm.

*The indicator NaCl solution* is prepared from 0.9 per cent NaCl solution from distilled water. It is desirable, but not necessary to have this $CO_2$-free. About 45 cc. of this solution are placed in a 50 cc. measuring flask and *exactly* 0.54 cc. of 0.06 per cent phenol red solution is added. 1 to 3 drops of 0.02 N NaOH are added to

bring the solution to pH 7.4 to 7.6, and the solution is diluted to 50 cc. with NaCl solution. (The saline solution must contain $\frac{54}{50}$ as much indicator as the standard, hence the 0.54 cc. of indicator to allow for the 0.4 cc. of blood.)

*The standard solutions* are prepared from Sörensen's phosphate at 0.05 pH intervals, using 0.10 cc. of the same indicator per 10 cc. of standard solution.

The phenol red stock solution is prepared in 0.1 per cent concentration by dissolving 0.1 gm. in 5.7 cc. of 0.05 N NaOH, adding about 80 cc. of distilled water, boiling, diluting to 100 cc., and filtering. Since phenol red preparations vary, it is best to determine what concentration of indicator gives, under the conditions used, the best difference in color.

*Paraffin oil.* If the mineral oil is too heavy, it interferes with both the blood flow from the incision and with measurement in the fine pipette. We use an oil of density 0.885.

The glass apparatus can be cleaned best in concentrated sulfuric acid saturated with bichromate.

## EXPERIMENTAL.

In adapting this method to capillary blood several questions had to be answered.

*Loss of $CO_2$ in Oil.*—The blood forming in small drops and sinking down slowly through the oil offered the maximum surface for loss of $CO_2$. An estimate of the extent of the loss was arrived at by testing the change in pH of a 0.03 N $NaHCO_3$ solution adjusted to pH 7.4 with $CO_2$. This is the concentration of $NaHCO_3$ in blood. The results of several such tests are given in Table I. It is evident that the loss is consistently such that pH increases about 0.03.

*Blood Clotting.*—Several types of funnel arrangements were made with the idea of drawing or allowing the blood to run into a tube for either sampling or centrifuging. In every case the extra contact with glass and the manipulation caused too rapid clotting. A funnel was adopted, closed at the bottom with either paraffin or in the flame, because that gave least clotting and was most convenient.

*Correction Factor for Infant Plasma and Serum.*—It was necessary to be certain that infant plasma or serum gave the same

correction factor *C* as adults. This question was raised because of the large species variation of unknown cause. Two series of experiments were carried out.

In each of the two following experiments about 7 cc. of serum, pooled from the bloods of four and three infants, were equilibrated

TABLE I.

*Loss of $CO_2$ in Oil.*

$BHCO_3 + H_2CO_3$ solution at 38° C. and at the pH indicated allowed to drop through oil. The time indicates the total time from the first to the last drop.

| Time. | Volume. | Initial pH. | pH after oil. | Difference. |
|---|---|---|---|---|
| *min.* | cc. | | | |
| 1 | 2 | 7.32 | 7.32 | 0 |
| 2 | 5 | 7.23 | 7.25 | +0.02 |
| 2 | 5 | 7.23 | 7.26 | +0.03 |
| 2 | 4 | 7.44 | 7.47 | +0.03 |
| 2 | 5 | 7.52 | 7.56 | +0.04 |
| 2½ | 5 | 7.45 | 7.48 | +0.03 |
| 4 | 5 | 7.51 | 7.56 | +0.05 |
| 4½ | 4 | 7.41 | 7.44 | +0.03 |
| 5 | 5 | 7.45 | 7.47 | +0.02 |
| 5 | 5 | 7.50 | 7.52 | +0.02 |
| Average | | | | +0.03 |

at known $CO_2$ tension, and electrometric and colorimetric pH determinations were made. The technique was precisely that used in working out the constants for the original method.

The results were as follows:

| Experiment No. | Electrometric 38° undiluted. | Colorimetric 38° diluted 21×. | "*C*" |
|---|---|---|---|
| | *pH* | *pH* | |
| 1 | 7.35 | 7.59 | 0.24 |
| 2 | 7.18 | 7.41 | 0.23 |

Hastings and Sendroy have recently reported that the reading of the 21× diluted plasma at 38° corresponds with the undiluted electrometric: that is, a correction is not necessary. If this is correct in all cases it affords a convenient way to check the cor-

rection used. We used it in this way, see Table II, putting a small thermometer through the rubber stopper and reading first at room temperature and then at 38°—both standards and solutions must be at the same temperature. Our results in these two series indicate that the correction of 0.23 is exact for children's blood and that with infants the serum and plasma correction is the same. Without a special bath at 38° we find it much more convenient to work at room temperature and to use the correction.

TABLE II.

Difference between pH readings corrected to 38° by factor 0.23 and readings made with standards and solutions at 38°.

| No. | | $pH_{20°} - 0.23$ | $pH_{38°}$ | Difference. |
|---|---|---|---|---|
| 24 | Plasma. | 7.53 | 7.53 | 0 |
| 25 | | 7.47 | 7.49 | +0.02 |
| 26 | | 7.44 | 7.45 | +0.01 |
| 27 | | 7.41 | 7.44 | +0.03 |
| 28 | | 7.32 | 7.33 | +0.01 |
| 29 | | 7.37 | 7.39 | +0.02 |
| 33 | | 7.39 | 7.42 | +0.03 |
| 24 | Serum. | 7.53 | 7.53 | 0 |
| 25 | | 7.47 | 7.49 | +0.02 |
| 32 | | 7.40 | 7.40 | 0 |
| 35 | | 7.44 | 7.42 | +0.02 |
| 36 | | 7.45 | 7.46 | +0.01 |
| 37 | | 7.39 | 7.39 | 0 |
| Average | | | | +0.015 |

*pH of Normal Plasma.*—As a further check we have run pH determinations on several children and normal adults. Here we used the correction factor of 0.26 (0.23 + 0.03). The results follow.

| | No. | Maximum. | Minimum. | Average. |
|---|---|---|---|---|
| | | *pH* | *pH* | *pH* |
| Infants | 25 | 7.50 | 7.34 | 7.41 |
| Adults | 15 | 7.44 | 7.34 | 7.40 |

The adults were healthy colleagues and nurses. Blood was taken from the ear without any previous preparation and under

ordinary respiration. The infants were all hospital patients under 1 year, selected from such light sicknesses as could have no influence on the normality of the blood pH. None was atrophic or had suffered from acute digestive disturbances, epilepsy, tetany, or rickets. The bloods were all taken 3 hours after a feeding.

These values for normal pH agree well with those previously reported by Cullen and Robinson (7.30 to 7.40), Bigwood (7.30 to 7.40), and Myers and Booher (7.34 to 7.43); all with the same technique. The average reported here is about 0.03 pH more alkaline than these values. This corresponds to the difference between the arterial blood used here and the venous blood used before. That "cutaneous" blood obtained in this manner represents arterial blood has been shown by Lundsgaard and Möller who have discussed the question thoroughly.

The higher maximum for infants is also to be expected for their pH must be subject to the influence of voluntary hyperpnea during crying.

## SUMMARY.

1. A simple technique is described for obtaining cutaneous (capillary) blood from infants and from adults, without loss of $CO_2$, and suitable for acid-base balance studies.

2. Cullen's colorimetric method for plasma, using Hawkin's modification for whole blood, is accurate for infant's blood.

3. The pH values obtained for both adults and infants with this combined technique are normal.

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# THE INTERFERING EFFECT OF GLYCEROL ON THE BIURET REACTION.*

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(Received for publication, March 13, 1925.)

A number of substances which interfere with the delicacy of the biuret reaction for protein have been described. For example, it is well known that if ammonium salts are present in a protein solution the expected violet color will not appear until after the ammonia is expelled by an excess of sodium hydroxide. This interference is due to the formation of the complex salt, $[Cu(NH_3)_4]SO_4 \cdot H_2O$, in which the copper is so bound that it cannot combine with the protein to produce the characteristic color. Other interfering substances are uric acid and creatinine in urine in excessive amount (Van Norman (1)) and paper and other forms of cellulose (Kantor and Gies (2)).

It is reasonable to expect that glycerol might interfere with the biuret reaction, since it holds copper hydroxide in solution. If the copper is bound by the glycerol in such form that it is not available for combination with protein, the biuret color will not appear. This has been found to be the case, and since mention is not made of this fact in text-books describing the reaction, it seemed worth while to discuss it briefly here. A consideration of this interference by glycerol is especially important in testing for protein in bacterial culture media where glycerol has been an ingredient. It is in this latter connection that the present study was made.

There is marked disagreement in the literature as to the production of protein by the tubercle bacillus in the fluid surrounding it in the course of its growth on non-protein media. Gly-

* Aided by a grant from the National Tuberculosis Association.



XUM

cerol is a practical necessity for the growth of *Bacillus tuberculosis*, and is invariably used in such media. As will be brought out below the biuret test in such media is not of great delicacy.

*Experiments.*

Two highly purified proteins, casein and gliadin, were dissolved and mixed with solutions of glycerol in order to determine the relative amount of weakening of the biuret color. In making the tests 1 cc. of glycerol solutions of varying strength was added to 1 cc. of protein solutions of increasing dilution. Then 1 cc. of a 10 per cent sodium hydroxide solution was added, and finally 0.2 cc. of a 0.5 per cent copper sulfate solution. The colors were then compared with those produced in corresponding tubes to which no glycerol had been added. The colors ranged from a definite pink in the stronger protein solutions through a deep purple to a faint violet in the weaker solutions, as the blue of the copper hydroxide became more prominent.

An arbitrary scale for designating the color was adopted, and by this means it was found that even 0.33 per cent of glycerol (1 cc. of a 1 per cent solution in the test as carried out above) reduces the color so that it corresponds with that of a glycerol-free solution containing only half the concentration of protein. 1 cc. of a 50 per cent solution of glycerol reduces the apparent protein concentration about 4-fold. The addition of more copper merely intensifies the blue in the solution.

Corresponding strengths of casein and gliadin give approximately the same amount of biuret color. The delicacy of the test approaches as a limit a dilution of 1:9,600. The inhibition of color development by glycerol is about the same for both proteins.

The statements made above for the effect of glycerol upon the biuret test on casein and gliadin hold true for a protein isolated from a tuberculin produced by the growth of the tubercle bacillus in a non-protein medium. The reduction in color by 16.66 per cent of glycerol is the same as with these two proteins; *i.e.*, the color developed corresponds to that found in a dilution of protein four times as great in glycerol-free solution. The decrease of color from the normal in the presence of 0.33 per

cent of glycerol is somewhat greater than in the case of casein and gliadin.

The color produced with 1 cc. of a 1:100 tuberculin protein solution is unreliable because of the interference of the yellow color of this protein. Similarly the color in the corresponding dilution of gliadin is unreliable because of the turbidity due to the insolubility of the protein in the alkali.

The tuberculin used for the preparation of the tuberculin protein of these experiments was produced from a synthetic medium described by Long (3), containing ammonium citrate. Since citrate may hold copper in solution (4), and since ammonium salts may interfere with the biuret test, the effect of ammonium citrate was tested. In 0.66 per cent concentration, or approximately that used in the medium mentioned, it was found to have no interfering action on the biuret reaction.

## SUMMARY.

Glycerol interferes markedly with the delicacy of the biuret test, presumably through its combining power for copper hydroxide. As small an amount as 0.33 per cent prevents the reaction in solutions of casein and gliadin of 1:9,600 concentration, and in solutions of tuberculin protein of 1:4,800 concentration, the limiting concentrations at which these proteins give a positive biuret test in the absence of glycerol. At a concentration of 16.66 per cent, glycerol reduces the apparent protein concentration 4-fold; *i.e.*, the color developed is weak, corresponding to that found in a dilution of protein four times as great in a solution containing no glycerol.

This inhibitory action of glycerol on the biuret test is of great significance in testing non-protein bacteriological culture media for the development of protein as a result of bacterial growth.

Ammonium citrate in concentrations corresponding to those used in culture media does not inhibit the development of the biuret color.

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# THE MECHANISM OF OXIDATION OF THYMINE.

## 4,5-DIHYDROXYHYDROTHYMINE (THYMINE GLYCOL).

By OSKAR BAUDISCH and DAVID DAVIDSON.

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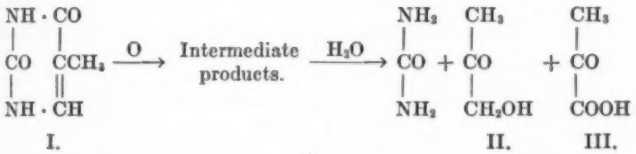
(Received for publication, April 29, 1925.)

Johnson and Baudisch (1) first observed that thymine (I) is readily attacked by ferrous bicarbonate plus air, forming products which are readily hydrolyzed to urea, acetole (II), and pyruvic acid (III).




I. II. III.

Baudisch and Bass (2) extended this observation and were able to show that when hydrogen peroxide alone, or hydrogen peroxide plus ferrous sulfate acted upon thymine the same products were formed as in the case with ferrous bicarbonate plus air. Furthermore, sodium pentacyano-aquo-ferroate (aquo salt) plus oxygen converted thymine to products leading only to urea and pyruvic acid, no acetole being formed, while sodium bicarbonate plus iodine yielded material which on hydrolysis produced only urea and acetole, no pyruvic acid being detectable. Bass (3) later demonstrated that under the influence of ultra-violet light oxygen was capable of attacking the thymine molecule in the presence of ferrous sulfate so that subsequent hydrolysis gave urea and pyruvic acid.

It was suggested by Baudisch and Bass that the first stage of the oxidation of thymine might be represented in the following manner.



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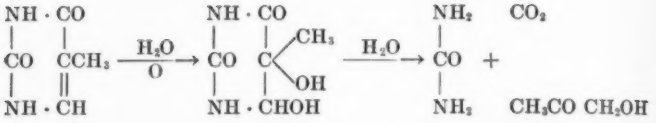
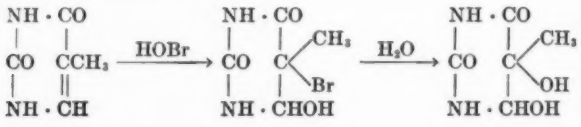



I. IV.

In order to test this hypothesis it was desirable to study the previously unknown thymine glycol (4,5-dihydroxyhydrothymine) (IV). We have succeeded in obtaining this substance in crystalline condition and shall report here upon its preparation and some of its properties.

*Preparation of Thymine Glycol.*

Thymine is readily converted (4) by bromine water into 5-bromo-4-hydroxyhydrothymine (V). Wheeler and Johnson (5) noted that bromohydroxyhydrothymine did not yield a colored salt when treated with $Ba(OH)_2$ but they did not investigate the reaction which occurs.

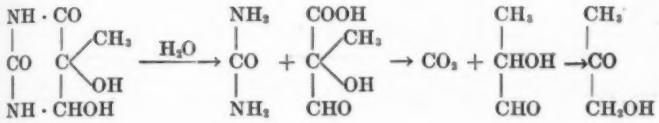



I. V. IV.

It is now found that bromohydroxyhydrothymine (V) is instantaneously attacked by alkalies [$Ba(OH)_2$, NaOH, $Ag_2O$] in the cold, the bromine being completely removed. The isolation of a crystalline product, however, succeeded only when silver oxide was employed, and in this case the reaction proceeded very smoothly and yielded pure thymine glycol to the extent of 70 per cent of the theory.

*Hydrolysis of Thymine Glycol.*

Thymine glycol is very susceptible to the action of alkalies. Boiling sodium bicarbonate solution decomposes it, urea and acetole being produced according to the reaction:



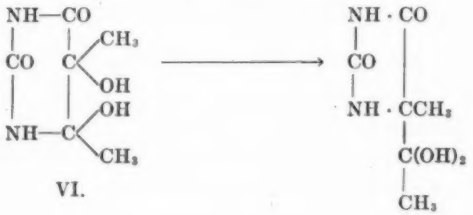
IV. IX. II.

When the hydrolysis is carried out with excess of $Ba(OH)_2$ solution, 1 molecule of $CO_2$ is *quantitatively* precipitated as $BaCO_3$.

It is to be emphasized that the introduction of two hydroxyl groups into the pyrimidine ring completely alters the stability of this cycle and renders it easily split into urea and an aliphatic residue. In the present case lactic aldehyde (IX) undoubtedly is the immediate product which results from the loss of $CO_2$ from $CH_3 \cdot C(OH)(COOH)CHO$, but as has been shown by Nef (6) and others, this aldehyde readily isomerizes into acetole, more especially in alkaline solution.

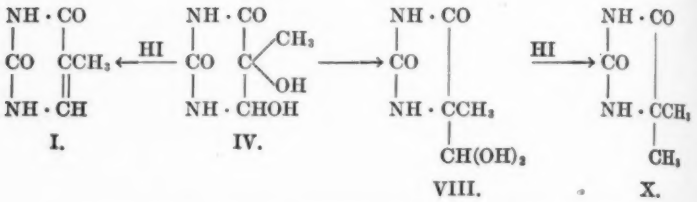
*Reduction of Thymine Glycol.*

In discussing glycols of 4-methylthymine and of N-methylated-4-methylthymine Behrend and his coworkers have assumed spontaneous rearrangement into 5-methyl-5-acetyl hydantoins; thus it is supposed that 4-methylthymine glycol (VI) really has but a transitory existence, being instantaneously transformed into 5-methyl-5-acetyl hydantoin hydrate (VII).



VI. VII.

If the conceptions outlined above are applied to thymine glycol it may be postulated that it, too, undergoes rapid conversion into 5-methyl-5-formyl hydantoin (VIII).

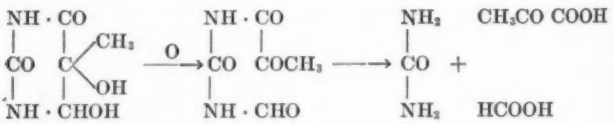


I. IV. VIII. X.

In order readily to distinguish between the two structures (IV) and (VIII) thymine glycol was submitted to reduction with hydriodic acid. By this treatment one may anticipate that thymine (I) will be formed from structure (IV) or 5,5-dimethyl hydantoin (X) from structure (VIII). Actually, thymine was obtained, thus conclusively demonstrating the simple relationship between thymine and thymine glycol; *i.e.*, the persistence of the pyrimidine ring in the oxidized compound.

*Oxidation of Thymine Glycol.*

Since no pyruvic acid is produced by the hydrolysis of thymine glycol it may be assumed that in the oxidation of thymine, pyruvic acid results from the hydrolysis of an oxidation product of thymine glycol. To test this hypothesis thymine glycol was subjected to oxidation by oxygen in the presence of aquo salt. Hydrolysis of the resulting solution now yielded a positive test for pyruvic acid. Although this progenitor of pyruvic acid was not isolated, we may conclude on theoretical grounds that the reaction proceeds through the formation of formyl pyruvic urea:



IV.

SUMMARY.

Thymine glycol has been prepared and its constitution confirmed.

It is quantitatively hydrolyzed to acetole, urea, and carbon dioxide.

On oxidation it yields a product which is hydrolyzed to pyruvic acid.

It may be assumed, therefore, that thymine glycol is produced in the oxidation of thymine.

## EXPERIMENTAL PART.

*Thymine* was prepared by the methods of Wheeler and McFarland (7).

*5-Bromo-4-Hydroxyhydrothymine.*—Jones' (4) method was modified as follows:

15 gm. of pulverized thymine were suspended in 150 cc. of water and 7.5 cc. of bromine added at once. The reaction proceeded rapidly in the cold with the separation of a voluminous precipitate. When the layer of bromine had disappeared the reaction mixture was heated on the steam bath until all was in solution. Excess of bromine was evidenced by the red color of the solution which was then filtered hot. On cooling pure bromohydroxyhydrothymine separated in large colorless prisms. Yield 20.4 gm.

*Analysis (Kjeldahl).*

| | | |
|---|---|---|
| $C_5H_7N_2O_3Br$. | Calculated. | N 12.57. |
| | Found. | " 12.47. |

Bromohydroxyhydrothymine is readily hydrolyzed by boiling with sodium bicarbonate into acetole and urea. With excess of $Ba(OH)_2$ it gradually precipitates $BaCO_3$.

### *4,5-Dihydroxyhydrothymine (Thymine Glycol).*

6.3 gm. of pulverized bromohydroxyhydrothymine and moist silver oxide, obtained from 6.0 gm. of silver nitrate (by precipitating in the hot with excess sodium hydroxide and washing thoroughly), were suspended in 150 cc. of water and shaken for 16 hours. The reaction mixture was then centrifuged, the opalescent supernatant liquor decanted, the precipitate suspended in water, and again centrifuged. The combined liquors were treated with hydrogen sulfide and, without filtering, evaporated to dryness under diminished pressure. The residue was taken up in 75 cc. of warm water and a little bone charcoal added. After filtering by

suction, the clear colorless filtrate was evaporated to crystallization under diminished pressure, when large colorless crystals of thymine glycol separated, were filtered off, and washed with a little cold water. Yield: 3.2 gm. (71 per cent of theory).

The compound melts with decomposition at about 220° and is readily soluble in water.

It reduces Fehling's solution and ammoniacal silver nitrate.

*Analysis.*

| | | |
|---|---|---|
| $C_5H_8N_2O_4$. | Calculated. | C 37.48, H 5.04, N 17.50. |
| | Found. | " 37.02, " 5.19, " 17.53, 17.31. |

*Hydrolysis of Thymine Glycol.*

*(A). By $NaHCO_3$.*

1 gm. of thymine glycol was distilled with 5 gm. of sodium bicarbonate and 100 cc. of water until about 65 cc. of distillate had been collected. The distillate was treated with an excess of phenylhydrazine and acetic acid. Precipitation of acetole phenylosazone began in the cold and was completed by warming on the water bath. It was recrystallized by dissolving it in warm benzene and diluting with ligroin. Melting point 150° (mixed melting point).

The residue from the distillation had a strong sweet odor, gave a characteristic precipitate of dixanthyl urea with xanthydrol, but no test for pyruvic acid with ortho-nitrobenzaldehyde (Baeyer and Drewsen (8)).

*(B). By $Ba(OH)_2$.*

This hydrolysis was carried out in a 300 cc. Claisen distilling bulb, attached to a condenser. A current of steam was passed through 100 cc. of boiling $Ba(OH)_2$ solution (saturated at 15°C.) contained in the bulb while a solution of 1 gm. of thymine glycol in 100 cc. of water was dropped into the flask through the second neck. $BaCO_3$ began to precipitate immediately while acetole distilled. The operation was complete in 15 to 20 minutes. The $BaCO_3$ was filtered off and dried at 110°. It weighed 1.24 gm. (theory, 1.23 gm.). The filtrate had a strong sweet odor and gave a characteristic precipitate of dixanthyl urea with xanthydrol.

The distillate reduced Fehling's solution and ammoniacal silver nitrate even in the cold. Acetole was identified by the method of Baudisch (9).

*Reduction of Thymine Glycol.*

1 gm. of pulverized thymine glycol was heated on the steam bath with 7.5 cc. of hydriodic acid (sp. gr. 1.7). The pyrimidine readily went into solution. As the heating was continued a crystalline product separated. After 45 minutes, heating was discontinued, the mixture was cooled, and 150 cc. of ether were added with enough acetone to produce a homogeneous solution. Filtration yielded 0.24 gm. of a nearly colorless product which on recrystallization from 10 cc. of boiling water deposited in brilliant plates characteristic of thymine. It did not melt below 300°, gave a green color with aquo salt and the following analysis.

*Analysis.*

| | | |
|---|---|---|
| $C_5H_6O_2N_2$. | Calculated. | N 22.23. |
| | Found. | " 22.48. |

*Oxidation of Thymine Glycol.*

0.2 gm. of thymine glycol and 0.2 gm. of sodium pentacyano-aquo-ferroate were dissolved in 50 cc. of water. Air was then bubbled through the solution for 20 hours. A portion of the solution on evaporation with sodium bicarbonate gave a strong test for pyruvic acid. Thymine glycol itself when evaporated with bicarbonate gives no test for pyruvic acid.

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# PROTEINS OF WHEAT BRAN.

## II. DISTRIBUTION OF NITROGEN, PERCENTAGES OF AMINO ACIDS AND OF FREE AMINO NITROGEN: A COMPARISON OF THE BRAN PROTEINS WITH THE CORRESPONDING PROTEINS OF WHEAT ENDOSPERM AND EMBRYO.

By D. BREESE JONES and C. E. F. GERSDORFF.

*(From the Protein Investigation Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.)*

(Received for publication, April 6, 1925.)

Approximately 22 per cent of the nitrogen of the wheat kernel resides in the seed coats or bran. This represents a vast amount of protein in the total wheat crop which is produced annually. Inasmuch as wheat is probably the most extensively used item in the food of civilized man, it is rather surprising that heretofore practically nothing has been known regarding the character and properties of the proteins of wheat bran. Results obtained by the use of bran in the practical feeding of animals have led to the general view that the bran proteins have a high nutritive value. It has been also amply demonstrated by feeding experiments with small animals that the proteins of the whole wheat kernel adequately supply the animal's dietary protein requirements, and that they are superior in this respect to the proteins of the endosperm, which constitute almost entirely the proteins of ordinary white wheat flour. From these considerations it appeared probable that the bran proteins are of such a character as to supplement nutritively those of the endosperm; that they are rich in those nutritionally essential amino acids in which the endosperm proteins are deficient. The results of our studies on the bran proteins have substantiated the above supposition.

The writers have previously shown (1) that the proteins of wheat bran consist essentially of a prolamin, a globulin, and an albumin. These proteins were obtained in yields of 5.35, 2.35, and 2.87 per



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cent, respectively, and were isolated from a hard wheat bran, from which nearly all of the other parts of the seed usually associated with commercial bran had been removed by a special treatment (1). The above percentages of protein which were isolated represent collectively 61.27 per cent of the total protein in the bran as calculated from its nitrogen content. Since not all of the nitrogen of the bran is protein nitrogen, the percentage of the total true protein isolated must be somewhat higher than the above figure cited.

Although the elementary composition of the bran proteins and some of their properties strongly indicated that these proteins differed from the corresponding proteins of the wheat endosperm and embryo, final conclusions on this point were deferred until further knowledge regarding them should be available.

A striking difference between the general character of the protein content of the wheat bran and that of the endosperm lies in the fact that approximately half of the protein of the endosperm consists of a glutelin (glutenin), while, on the other hand, no protein of the glutelin type was found in the bran.

In this paper we are now presenting data showing the distribution of nitrogen and the percentages of the dibasic amino acids in the bran proteins as determined by the Van Slyke method. The tryptophane, cystine, and tyrosine contents of these proteins have also been estimated by means of colorimetric methods.

The results of the analyses of the bran proteins show that these proteins contain relatively high percentages of the basic amino acids. These percentages, expressed in terms of the ash- and moisture-free proteins, are summarized in Table I. The quantity of basic amino acids in the globulin, particularly arginine and lysine, exceeds that of most other vegetable proteins which have been studied. The high percentages of tryptophane and cystine found in the albumin are also of further particular interest. The relatively large quantities of the so called nutritionally essential amino acids, such as lysine, tryptophane, and cystine, found in the wheat bran proteins are especially significant from the standpoint of their nutritive value. Feeding experiments with rats are in progress to supplement these chemical data regarding the nutritive value of the bran proteins, and to ascertain the availability to the animals of the amino acids present.

*Comparison of the Bran Proteins with the Corresponding Proteins of the Endosperm and Embryo.*

Whether we look upon bran as a valuable source of protein which should be included in wheat products used for human consumption, or as more suitable to be used as a protein concentrate for the feeding of domestic animals, it is of importance to know how the bran proteins compare with those of the other parts of the seed, and to ascertain their amino acid composition, particularly those amino acids which have been shown to be essential for the normal nutrition of animals.

TABLE I.

*Some Amino Acids of the Proteins of Wheat Bran* (Expressed as Percentage of the Ash- and Moisture-Free Proteins.*

| Amino acids. | Prolamin. | Globulin. | Albumin. |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| Cystine | 1.65 | 1.38 | 2.80 |
| | 2.29† | 1.52† | 3.29† |
| Arginine | 4.41 | 14.13 | 10.04 |
| Histidine | 0.84 | 2.76 | 2.57 |
| Lysine | 2.45 | 11.84 | 4.51 |
| Tyrosine† | 3.38 | 3.69 | 4.20 |
| Tryptophane‡ | 1.37 | 2.85 | 4.76 |

* The percentages of the amino acids given in Table I, with the exceptions noted, were calculated from the results obtained by the Van Slyke method of analysis.

† Determined by the colorimetric methods of Folin and Looney (8).

‡ Determined by the colorimetric method of May and Rose, with modifications (10).

For the purpose of comparison the figures expressing the elementary composition, distribution of nitrogen, percentages of amino acids, and the free amino nitrogen of the bran proteins and of the corresponding proteins of the wheat endosperm and embryo have been assembled in Table II. The data given for the proteins of the endosperm and embryo are mostly figures which have been published by Osborne and associates. All of the percentages, however, given in the table for tryptophane, cystine, free amino nitrogen, and tyrosine, with the exception of the tyrosine figure for leucosin, were determined by the writers.

*Prolamin.*—A notable difference between the alcohol-soluble protein of the bran and that of the endosperm (gliadin) is shown by their elementary composition, particularly with reference to carbon and nitrogen. That the relatively low percentage of nitrogen in the bran prolamin is characteristic of this protein is indicated by the concordant results obtained by nitrogen determinations made on five different samples of the prolamin prepared by different methods and under varying conditions.[1]

The bran prolamin was found to contain about twice as much basic nitrogen as that recorded for gliadin, although its total nitrogen content was about 2 per cent lower. This difference in the nitrogen distribution of the two prolamins is to be accounted for by the higher percentages of the basic amino acids found in the bran prolamin as compared with those of gliadin. Twice as much lysine was found in the bran prolamin as that recorded by Osborne, Van Slyke, Leavenworth, and Vinograd (2) for gliadin, and also a much higher content of arginine. The histidine content of the bran prolamin, on the other hand, was found to be much lower than that given for gliadin.

The estimation of tryptophane, tyrosine, and cystine in the bran prolamin, and in a sample of wheat gliadin especially prepared by the writers, gave closely agreeing results.

The free amino nitrogen of the bran prolamin was found to be practically three times that of gliadin.

*Albumin.*—About 2.9 per cent of wheat bran consists of an albumin which separates as a flocculent coagulum when its aqueous (slightly acidified with acetic acid) solution is slowly heated to about 60°C. When heated in 4 per cent sodium chloride solution it coagulates at about 65°C. The albumin can be obtained nearly white when dried in the usual way, and is free from phosphorus.

Osborne (3), working with commercial wheat embryo—a mixture consisting largely of embryo and containing more or less of the endosperm and fine bran—isolated somewhat more than 10

[1] A description of the preparation and composition of these samples is given in a previous publication (1). The figures showing the elementary composition of the bran prolamin given in Table II are averages obtained by duplicate analyses made on the five preparations.

per cent of an albumin (leucosin), which was found to coagulate at 55–60°C.

Aside from the difference in their coagulation points, the two albumins also differ with respect to their composition as

TABLE II.

*Analytical Data Shown for Comparison of the Prolamin, Albumin, and Globulin of Wheat Bran with the Corresponding Proteins of Other Parts of the Wheat Kernel.**

(The values are expressed as percentages of the proteins).

| Determinations. | Bran prolamin. | Gliadin. | Bran albumin. | Leucosin. | Bran globulin. | Embryo globulin. |
|---|---|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Carbon | 54.25 | 52.72 | 53.21 | 53.02 | 53.43 | 51.03 |
| Hydrogen | 6.75 | 6.86 | 6.71 | 6.84 | 7.40 | 6.85 |
| Nitrogen | 15.35 | 17.66 | 15.42 | 16.80 | 17.76 | 18.39 |
| Sulfur | 1.35 | 1.14 | 1.35 | 1.28 | 0.91 | 0.69 |
| Amide N | 3.58 | 5.12 | 1.20 | 1.16 | 1.06 | 1.42 |
| Humin N | 0.18 | 0.07 | 0.52 | 0.43 | 0.29 | 0.28 |
| Basic N | 2.30 | 1.00 | 5.12 | 3.50 | 7.73 | 6.83 |
| Non-basic N | 9.53 | 12.25 | 8.92 | 11.83 | 8.82 | 9.82 |
| Cystine | 2.29 | 1.76 | 3.29 | | 1.52 | |
| Arginine | 4.41 | 2.97 | 10.04 | 5.94 | 14.13 | |
| Histidine | 0.84 | 2.19 | 2.57 | 2.83 | 2.76 | |
| Lysine | 2.45 | 1.21 | 4.51 | 2.75 | 11.84 | |
| Tryptophane | 1.37 | 1.09 | 4.76 | | 2.85 | |
| Tyrosine | 3.38 | 3.30 | 4.20 | 3.34 | 3.69 | |
| Free amino N† | 1.88 | 0.65 | 2.84 | | 6.59 | |

* The figures given for gliadin, leucosin, and the globulin of the embryo are those obtained by Osborne and associates (2, 5), with the following exceptions, which were determined by the writers: All of the percentages given for cystine, tryptophane, and free amino nitrogen, and the tyrosine figure for gliadin.

† Expressed as percentage of the total nitrogen.

is shown by the data in Table II. A marked difference is shown by their nitrogen content. Again, as in the case of the prolamins, the bran albumin, although containing decidedly less total nitrogen than does leucosin, contains a much higher percentage of

basic nitrogen. The percentages of carbon, hydrogen, and sulfur in the two albumins, however, are in close agreement.

The percentages of the basic amino acids in the bran albumin were calculated from the results obtained by the Van Slyke method of analysis, while those given for leucosin as found by Osborne and Clapp (4) were determined by isolation according to the method of Kossel and Kutcher. Consequently we do not have a satisfactory basis upon which to make a fair comparison of the proportions of the basic amino acids in the two albumins, since the values obtained by the Van Slyke method are maximal and those obtained by Kossel and Kutcher's method are minimal. However, the percentages found for arginine by the two methods are usually in fairly close agreement. The great difference in the percentage of arginine found in the bran albumin as compared with that given for leucosin in Table II further strongly indicates that we are dealing here with two different proteins.

The percentage of tyrosine found in the bran albumin (4.2 per cent) was determined by means of Folin and Looney's colorimetric method. Here again a satisfactory basis of comparison is lacking, because the figure given by Osborne and Clapp (4) for tyrosine in leucosin (3.34 per cent) was based on the weight of the pure isolated amino acid. The difficulty involved in quantitatively separating tyrosine from mixtures of other amino acids is well known. It is therefore probable that no significant difference exists in the percentages of tyrosine in the two albumins.

*Globulin.*—Osborne and Voorhees (5) obtained from a "straight" wheat flour about 0.6 per cent of a globulin. Later, Osborne and Campbell (6) found that about 5 per cent of commercial wheat embryo consists of globulin. They found no difference in the composition of the globulins obtained from these two sources.

The bran globulin is precipitated from a 4 per cent sodium chloride solution by the addition of ammonium sulfate until 0.4 to 0.65 of saturation is reached. It coagulates in a slightly acidified 4 per cent sodium chloride solution at 95°C. Regarding the globulin of the embryo, Osborne states (3) that it is precipitated by saturating its solutions with magnesium sulfate, but not with sodium chloride. Dissolved in 10 per cent sodium chloride solution, it is partly but not completely coagulated at temperatures below 100°C.

A marked difference between the bran globulin and the globulin isolated by Osborne and Campbell is indicated by their elementary composition as shown in Table II. The bran globulin is also higher in basic nitrogen.

Especially noteworthy in connection with the bran globulin are the high percentages of arginine and lysine (Tables I and V). No data showing the amino acid composition of the embryo globulin are available for comparison. The bran globulin was found to be free from phosphorus.

From the comparative data which have been presented it is clear that there can be but little doubt, if any, that the proteins obtained from wheat bran are distinctly different from the corresponding proteins of the embryo and endosperm. Further studies of the bran proteins are in progress.

## EXPERIMENTAL.

The bran proteins used for the analyses herein described were prepared from bran which originally came mostly from 75 per cent Dark 1 Northern Spring Wheat and 25 per cent of 2 Hard Kansas Wheat. A detailed description of how the bran was prepared and of the separation and elementary composition of the proteins has been given in a previous publication (1).

### *Analyses of the Proteins by the Van Slyke Method.*

Duplicate samples of 3 gm. each of the bran proteins were hydrolyzed by boiling for about 30 hours with 100 cc. of 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed in the usual way with a mixture of ether and amyl alcohol. The results of the analyses are given in Tables I, III, IV, V, and VI.

The figures expressing the percentages of the basic amino acids in the proteins, as calculated from the results obtained by the Van Slyke analyses, are included in Table I.

The distribution of nitrogen in the proteins as calculated in terms of percentage of the proteins is given in Table VI.

TABLE III.

*Distribution of Nitrogen in the Wheat Bran Prolamin as Determined by the Van Slyke Method.**

Sample I, ash- and moisture-free, 2.7390 gm. protein, 0.4262 gm. nitrogen.†
" II, " " " 2.7390 " " 0.4262 " " †

| | I | II | I | II | Average. |
|---|---|---|---|---|---|
| | gm. | gm. | per cent | per cent | per cent |
| Amide N | 0.0982 | 0.0978 | 23.04 | 22.95 | 23.00 |
| Humin N adsorbed by lime | 0.0042 | 0.0038 | 0.99 | 0.89 | 0.94 |
| " " in ether-amyl alcohol extract. | 0.0009 | 0.0012 | 0.21 | 0.28 | 0.24 |
| Cystine N | 0.0051 | 0.0054 | 1.20 | 1.27 | 1.23 |
| Arginine N | 0.0387 | 0.0390 | 9.08 | 9.15 | 9.12 |
| Histidine N | 0.0054 | 0.0070 | 1.27 | 1.64 | 1.45 |
| Lysine N | 0.0136 | 0.0121 | 3.19 | 2.84 | 3.02 |
| Amino N of filtrate | 0.2399 | 0.2385 | 56.29 | 55.96 | 56.13 |
| Non-amino N of filtrate | 0.0224 | 0.0210 | 5.26 | 4.93 | 5.09 |
| Total N regained | 0.4284 | 0.4258 | 100.53 | 99.91 | 100.22 |

* Nitrogen figures corrected for the solubilities of the phosphotungstates of the bases.

† Nitrogen content of the protein, 15.56 per cent.

TABLE IV.

*Distribution of Nitrogen in the Wheat Bran Albumin as Determined by the Van Slyke Method.**

Sample I, ash- and moisture-free, 2.7225 gm. protein, 0.4250 gm. nitrogen.†
" II, " " " 2.7225 " " 0.4250 " " †

| | I | II | I | II | Average. |
|---|---|---|---|---|---|
| | gm. | gm. | per cent | per cent | per cent |
| Amide N | 0.0325 | 0.0326 | 7.65 | 7.67 | 7.66 |
| Humin N adsorbed by lime | 0.0130 | 0.0133 | 3.06 | 3.13 | 3.09 |
| " " in ether-amyl alcohol extract. | 0.0010 | 0.0009 | 0.24 | 0.21 | 0.22 |
| Cystine N | 0.0087 | 0.0091 | 2.05 | 2.14 | 2.10 |
| Arginine N | 0.0881 | 0.0878 | 20.73 | 20.66 | 20.70 |
| Histidine N | 0.0196 | 0.0182 | 4.61 | 4.28 | 4.45 |
| Lysine N | 0.0236 | 0.0235 | 5.55 | 5.53 | 5.54 |
| Amino N of filtrate | 0.2388 | 0.2396 | 56.19 | 56.38 | 56.28 |
| Non-amino N of filtrate | 0.0031 | 0.0037 | 0.73 | 0.87 | 0.80 |
| Total N regained | 0.4284 | 0.4287 | 100.81 | 100.87 | 100.84 |

* Nitrogen figures corrected for the solubilities of the phosphotungstates of the bases.

† Nitrogen content of the protein, 15.61 per cent.

TABLE V.

*Distribution of Nitrogen in the Wheat Bran Globulin as Determined by the Van Slyke Method.**

Sample I, ash- and moisture-free, 2.8344 gm. protein, 0.5105 gm. nitrogen.†
" II, " " " 2.8344 " " 0.5105 " " †

| | I | II | I | II | Average. |
|---|---|---|---|---|---|
| | *gm.* | *gm.* | *per cent* | *per cent* | *per cent* |
| Amide N | 0.0301 | 0.0300 | 5.90 | 5.87 | 5.89 |
| Humin N adsorbed by lime | 0.0065 | 0.0070 | 1.27 | 1.37 | 1.32 |
| " " in ether-amyl alcohol extract. | 0.0013 | 0.0015 | 0.25 | 0.29 | 0.27 |
| Cystine N | 0.0046 | 0.0045 | 0.90 | 0.88 | 0.89 |
| Arginine N | 0.1289 | 0.1289 | 25.25 | 25.25 | 25.25 |
| Histidine N | 0.0209 | 0.0215 | 4.09 | 4.21 | 4.15 |
| Lysine N | 0.0642 | 0.0644 | 12.58 | 12.62 | 12.60 |
| Amino N of filtrate | 0.2358 | 0.2353 | 46.19 | 46.09 | 46.14 |
| Non-amino N of filtrate | 0.0146 | 0.0144 | 2.86 | 2.83 | 2.84 |
| Total N regained | 0.5069 | 0.5075 | 99.29 | 99.41 | 99.35 |

* Nitrogen figures corrected for solubilities of the phosphotungstates of the bases.

† Nitrogen content of the protein, 18.01 per cent.

TABLE VI.

*Distribution of Nitrogen in the Wheat Bran Proteins as Determined from the Van Slyke Analyses in Terms of Percentage of the Proteins.*

| Nitrogen. | Prolamin.* | Globulin.† | Albumin.‡ |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| Amide | 3.58 | 1.06 | 1.20 |
| Humin | 0.18 | 0.29 | 0.52 |
| Basic | 2.30 | 7.73 | 5.12 |
| Non-basic | 9.53 | 8.82 | 8.92 |
| Total | 15.59 | 17.90 | 15.76 |

* Nitrogen content 15.56 per cent.

† Nitrogen content 18.01 per cent.

‡ Nitrogen content 15.61 per cent.

*Free Amino Nitrogen.*

Quantities equivalent to 0.75 gm. of the moisture-free bran proteins and also of gliadin were dissolved in 2.5 cc. of glacial

acetic acid plus 1 cc. of distilled water, and the solutions diluted with water to 100 cc. 2 cc. of each of the protein solutions in duplicates were used for the determination of total nitrogen and amino nitrogen. Caprylic alcohol was used to prevent foaming, and the larger reaction bulb of the Van Slyke apparatus was used in combination with the micro gas burette. The results are given in Table VII.

It has been shown by Van Slyke and Birchard (7), and others, that the free amino nitrogen of many proteins corresponds closely to one-half of the lysine nitrogen. This relationship was found to hold true both in the case of the bran proteins and in that of

TABLE VII.

*Free Amino Nitrogen of the Wheat Bran Proteins and of Wheat Gliadin.*

| Protein. | Total N in 2 cc. | N gas from 2 cc. | Pressure. | Temperature. | Amino N in 2 cc. | Ratio of amino N to total N. | One-half of the lysine N, as determined by the Van Slyke method. |
|---|---|---|---|---|---|---|---|
| | *mg.* | *cc.* | *mm.* | *°C.* | *mg.* | *per cent* | *per cent* |
| Prolamin (bran)..... | 9.48 | 0.33 | 762 | 30 | 0.1786 | 1.88 | 1.51 |
| Albumin ( " )..... | 10.01 | 0.53 | 762 | 31 | 0.2841 | 2.84 | 2.77 |
| Globulin ( " )..... | 7.23 | 0.88 | 762.1 | 30 | 0.4765 | 6.59 | 6.30 |
| Gliadin (wheat)..... | 7.44 | 0.09 | 762 | 30 | 0.0487 | 0.65 | 0.66* |

* Calculated from the results obtained by Osborne and associates.

gliadin. It is noted that the ratio of free amino nitrogen to total nitrogen in the case of the bran prolamin is nearly three times that found in gliadin.

*Cystine, Tyrosine, and Tryptophane Estimations.*

The cystine, tyrosine, and tryptophane contents of the bran proteins, and also of gliadin, were estimated colorimetrically. Folin and Looney's method (8) was used for cystine and tyrosine, and that of May and Rose (9) for tryptophane. A detailed account of these estimations has been given in a recent publication (10). These results are here incorporated in Tables I and II.

SUMMARY.

The proteins of wheat bran, consisting essentially of a prolamin, a globulin, and an albumin, have now been analyzed by the Van Slyke method, and the distribution of nitrogen and the percentages of the basic amino acids have been estimated. The percentages of tyrosine, tryptophane, and cystine have also been estimated by colorimetric methods. The wheat bran proteins, in general, are characterized by a high content of the basic amino acids. This is particularly true of the albumin and globulin. The quantity of basic amino acids found in the globulin exceeds that of most other vegetable proteins which have been studied. The following percentages of amino acids were found: (1) In the *prolamin;* cystine 2.29, arginine 4.41, histidine 0.84, lysine 2.45, tryptophane 1.37, and tyrosine 3.38; (2) in the *albumin;* cystine 3.29, arginine 10.04, histidine 2.57, lysine 4.51, tryptophane 4.76, and tyrosine 4.20; and (3) in the *globulin;* cystine 1.52, arginine 14.13, histidine 2.76, lysine 11.84, tryptophane 2.85, and tyrosine 3.69.

Marked differences were found between the bran proteins and the corresponding proteins of the wheat embryo and endosperm, both with regard to their elementary composition, distribution of nitrogen, and amino acid content.

The significance of the high percentages of the so called nutritionally essential amino acids found is pointed out with respect to the nutritive value of the bran proteins.

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# A MICRO METHOD FOR DETERMINING NITROGEN.

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(Received for publication, March 24, 1925.)

One cannot expect any further contribution on the Kjeldahl method to be entirely novel, but, in a determination so often repeated, improvements are still urgent, such as the reduction of the number of manipulations, of the time, or of the amount of sample.

We have found that the total nitrogen of a physiological fluid or some given fraction thereof may be estimated on minute samples within 15 or 20 minutes by digesting with a special acid mixture of high oxidizing power and then Nesslerizing directly, thus avoiding the usual processes of distillation or aeration.

A special digestion tube is used, made from a Pyrex test-tube 25 × 250 mm. whose bottom is drawn to an obtuse point like that of the usual 15 cc. centrifuge tubes. This tapering end is bent at an angle of about 20 degrees to the long axis of the tube. The acid mixture consists of 100 cc. of concentrated sulfuric acid and 160 cc. of 60 per cent perchloric acid made up to 500 cc. with ammonia-free water.

The procedure is briefly: Measure a sample judged to contain from 0.25 to 2.0 mg. of nitrogen into the special Pyrex tube; add 1 cc. of acid mixture and a solid glass bead to minimize bumping; hold the tube over the free flame of a micro burner in such a way as to expose a maximum of the liquid's surface; boil until the mixture becomes dark colored and heavy fumes evolve; add 3 drops of hydrogen peroxide solution; insert a small funnel into the mouth of the digestion tube and turn it so as to expose a minimum of surface of the liquid; heat in such a manner that the liquid will simmer; when digestion is nearly complete, as shown by the disappearance of nearly all of the brownish color, add 3 drops of hydrogen perox-



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ide[1], to destroy the last trace of color; heat from 3 to 5 minutes after it is clear; add 3 or 4 cc. of water, add 8 cc. of 2 N sodium hydroxide, dilute to 50 cc., and mix; add 5 cc. of Nessler's reagent[2] diluted to 25 cc. and mix; dilute to the 80 or 100 cc. mark; match against a Nesslerized solution of 0.5, 1.0, or 1.5 mg. of ammonium sulfate per cc.

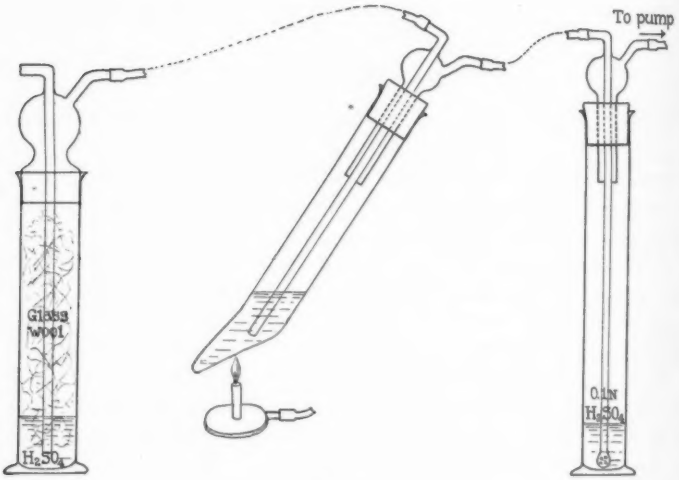


FIG. 1.

Hydrogen peroxide was employed in micro Kjeldahl determinations in V. C. Myers' laboratory before it had been mentioned in the literature as an aid in Kjeldahl digestion. Perchloric acid

[1] The hydrogen peroxide is "perhydrol" diluted 1 to 5. In the simplest cases, such as urinary nitrogen determinations, the hydrogen peroxide may be omitted. The hydrogen peroxide hastens the oxidation, and it helps in managing frothy digestions.

[2] In this laboratory the following quantities are used in making up 1 liter of Nessler's solution: $HgI_2$, 100 gm.; KI, 70 gm.; NaOH, 100 gm. Many formulas for making up Nessler's solution are to be found in the literature. Inasmuch as the degree of alkalinity affects the color value, the alkali should be kept constant within narrow limits (about 2 cc. of 0.1 N) by adhering to some one formula. This also applies to the neutralization of the digested sample.

was added to the regular Kjeldahl-Gunning macro procedure in 1921.[3] Direct Nesslerization has been described by Folin and Denis[4] and by Wong.[5]

TABLE I.

| Sample. | Calculated nitrogen. | Nitrogen by Kjeldahl method. Macro. | Nitrogen by Kjeldahl method. Micro.* | Nitrogen by Present method. Micro. |
|---|---|---|---|---|
| | *mg. per cc.* | *mg. per cc.* | *mg. per cc.* | *mg. per cc.* |
| Ammonium sulfate | 2.0 | 1.94 | 2.09 | 1.91 |
| Urea | 0.7 | | 0.67 | 0.68 |
| | 6.66 | | | 7.01 |
| Uric acid | 0.68 | | | 0.75 |
| | 1.00 | | | 0.96 |
| | 0.67 | | | 0.69 |
| Creatinine | 0.84 | | 0.63 | 0.74 |
| | 1.48 | | | 1.06 |
| | 0.74 | | 0.66 | 0.50 |
| Indole | 3.00 | | | 3.06 |
| Ornithuric acid† | 2.00 | | | 1.82 |
| Allantoin | 0.98 | | | 1.04 |
| *o*-Aminobenzoic acid | 1.36 | | | 1.44 |
| Glycocoll | 1.00 | 1.03 | | 0.97 |
| Urine | | 4.2 | | 4.2 |
| | | 3.2 | | 3.5 |
| Serum | | 9.9 | | 10.4 |
| | | 10.7 | | 10.5 |
| | | 10.2 | | 10.3 |
| | | 10.7 | | 10.7 |
| | | 10.4 | | 10.3 |
| | | 10.7 | | 10.6 |
| | | 10.4 | | 10.0 |
| Blood | | | 25.9 | 26.5 |
| | | 23.5 | | 24.0 |
| Phenylacetyl glutamine urea, *per cent.* | | 17.2 | 17.3 | 17.1 |

* Folin-Farmer method as given by Myers.
† Purity of preparation in question.

[3] Parker, J. G., and Terrell, J. T., *J. Soc. Leather Trades Chem.*, 1921, v, 380. Mears, B., and Hussey, R. E., *J. Ind. and Eng. Chem.*, 1921, xiii, 1054.
[4] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 486.
[5] Wong, S. Y., *J. Biol. Chem.*, 1923, lv, 427.

The time required for digestion varies with the nature of the sample, from 2 minutes after clear for a diluted urine, to more than 8 minutes for blood protein coagulum, or a fatty semisolid sample. Boiling off water requires 1 minute per cc. The time of heating after clear depends on the nature of the substances present and can best be left to the judgment of the individual analyst who will be guided in this by his experience with macro Kjeldahl operations or by a few preliminary tests. To obtain a blank on the reagent 1 cc. of the 1.0 mg. standard ammonium sulfate solution and a few grains of cane-sugar are digested as if this were a sample of urine. The analyst may encounter samples of too low a nitrogen content to warrant diluting, and rich in substances which make a viscid, frothy mess difficult to digest. It is then better to give up the advantage of direct Nesslerization. Double or treble the amount of acid mixture, use hydrogen peroxide more liberally, dilute the digested sample to 10 or 12 cc., insert a stopper with aeration tubes into the mouth of the digestion tube, connect with a sulfuric acid scrubber and a receiving cylinder containing 2 cc. of 0.1 N $H_2SO_4$, aerate and heat the digestion tube with a micro burner. By this combination of aeration and distillation (see Fig. 1) the ammonia will be transferred to the receiving cylinder in about 3 minutes. It is then Nesslerized in the usual way.

This procedure will give all the nitrogen expected from regular Kjeldahl methods. It will not give all of the guanidine and creatinine nitrogen. Excellent checks were obtained on known quantities of amino acids, cyclic amino compounds, urea, uric acid, and indole. Physiological fluids which had been analyzed by both macro and micro Kjeldahl digestion and distillation methods gave concordant results. Some of these are given in Table I.

# THE GLOBULINS OF THE JACK BEAN (CANAVALIA ENSIFORMIS).

## II. THE CONTENT OF CYSTINE, TYROSINE, AND TRYPTOPHANE.

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(Received for publication, March 24, 1925.)

During the past 2 years the authors have spent a large amount of time attempting to separate jack bean urease from the three globulins with which it is associated. As a result of this work a considerable amount of information has been gained concerning the properties of the jack bean globulins and methods for their purification. This paper is to be considered a continuation of the work published by the senior author in 1919.[1]

In view of the apparent protein nature of urease we have determined the percentages of cystine, tyrosine, and tryptophane in the globulins in an attempt to show that purified urease is, or is associated with, an entirely different protein. We have used the methods of Folin and Looney.[2] Some of our analytical results have been satisfactory while others have not been entirely satisfactory, although they are probably sufficient to serve the purpose for which they were intended. In those cases where the amino acid percentage is small and where it is necessary to take as much as 8 or 10 cc. of the hydrolysate for the development of the color we have found that the humin present interferes very seriously with the colorimetric comparison. The jack bean contains a colloidal pentosan which has markedly acid properties and which is very difficult to remove from the proteins. We have found that when this is boiled with 20 per cent sulfuric acid it yields a nonvolatile substance that reduces the molybdenum-free uric acid

[1] Sumner, J. B., *J. Biol. Chem.*, 1919, xxxvii, 137.

[2] Folin, O., and Looney, J. M., *J. Biol. Chem.*, 1922, li, 421.

reagent of Folin and Trimble[3] in the absence of sodium sulfite and consequently interferes with the Folin and Looney method for cystine. The same substance is produced from arabinose and from cherry gum. Fructose yields a slight amount of a similar substance, while glucose does not. Of the three jack bean globulins we are able to obtain one practically free from the pentosan by recrystallizing; this is concanavalin B. We have not been able to remove all of the pentosan from the other globulins by repeated solution and reprecipitation by dialysis. But we have found that the pentosan can be removed by adding to the solution of the globulins a large amount of neutral phosphate solution and sodium chloride and then alcohol to 30 per cent. After standing for some time the pentosan together with a small amount of protein is precipitated and can be filtered or centrifuged off. The globulin is then precipitated by dialysis. The determination of cystine by the Folin-Looney procedure gives satisfactory results when the amount of cystine is high but not when the amount of cystine is low. Some of the reasons for this are: (1) It is difficult to obtain a perfect blank as color is developed on adding the uric acid reagent to the carbonate-sulfite mixture even if great care is taken to rotate the solution while adding the uric acid reagent. (2) The amount of acid added with the hydrolysate makes a difference in the stability of the blue color. (3) With small quantities of cystine the solution is likely to become turbid. This is prevented somewhat by the presence of sodium bicarbonate. (4) For small amounts of cystine 3 cc. of the uric acid reagent are too much to use, 1 cc. is much better. Using 1 cc. the solution is not likely to become turbid and the amount of color produced by a blank is considerably less. When large amounts of cystine are present it is of course necessary to use 3 cc. of the uric acid reagent.

Recently Jones, Gersdorff, and Moeller[4] gave figures for the percentages of cystine and tryptophane in canavalin and concanavalin ($\alpha$-globulin). They have used the method of Folin and Looney[2] for cystine and the method of May and Rose[5] for the

[3] Folin, O., and Trimble, H., *J. Biol. Chem.*, 1924, lx, 473.

[4] Jones, D. B., Gersdorff, C. E. F., and Moeller, O., *J. Biol. Chem.*, 1924-25, lxii, 183.

[5] May, C. E., and Rose, E. R., *J. Biol. Chem.*, 1922, liv, 213.

determination of tryptophane. Their figures differ considerably from ours except in one instance, making it appear that if the methods of analysis are reliable the proteins used by Jones, Gersdorff, and Moeller were of a different degree of purity from ours. Inasmuch as they have used material fractionally precipitated by ammonium sulfate[6] instead of following the method given by one of us[1] this conclusion is inevitable. We give comparative figures below, also figures for concanavalin B and for the tyrosine content of the three globulins. Our figures are on an ash-free basis. The concanavalin A was recrystallized but once and contained a small amount of pentosan. The cystine determination has been repeated with concanavalin A that had been purified until practically free from pentosan. The canavalin used in the determination of tyrosine and tryptophane contained a small amount of pentosan but the determination of cystine was carried out with canavalin that was absolutely carbohydrate-free. See Table I.

TABLE I.

*Percentage of Amino Acids.*

| Protein. | Tryptophane. | | Cystine. | | Tyrosine. |
|---|---|---|---|---|---|
| | Authors' figures. | Jones, Gersdorff, and Moeller's figures. | Authors' figures. | Jones, Gersdorff, and Moeller's figures. | Authors' figures. |
| Canavalin | 0.24 | 0.21 | 1.0 | 0.40 | 5.5 |
| Concanavalin A | 2.2 | 3.36 | 0.4 | 1.57 | 5.2 |
| " B | 2.3 | | 3.2 | | 9.4 |

While we believe that our results for tryptophane and tyrosine, and for cystine in the case of concanavalin B, are essentially correct, we think that the figures for cystine in canavalin and concanavalin A are only approximate. We have repeated the determination a number of times. We do not believe that the figures of Jones, Gersdorff, and Moeller, showing concanavalin A to contain four times as much cystine as canavalin, are correct because of the fact that canavalin gives a fairly strong test for unoxidized sulfur when boiled with lead acetate and sodium hydroxide while concanavalin A gives almost no color at all.

[6] Jones, D. B., and Johns, C. O., *J. Biol. Chem.*, 1916–17, xxviii, 67.

Below are given improved methods for the purification of the three globulins.

*Concanavalin B.*—This globulin is present in the jack bean in very small amount. We have prepared it as a by-product in our method for the purification of urease. One mixes 2 liters of 30 to 35 per cent alcohol with 1 kilo of meal for ½ hour. The extract is pressed out from cheese-cloth in a tincture press, centrifuged as clear as possible, and allowed to stand in a liter graduate overnight at −10°C. The supernatant liquid is then syphoned off and the precipitate is centrifuged off while cold in cold centrifuge tubes and holders. Upon stirring up the precipitate with a little dilute neutral phosphate solution the needles of concanavalin B separate out quantitatively within a short time and are centrifuged off and washed with 2 per cent salt solution. The needles are recrystallized twice by dissolving in 10 per cent sodium chloride solution and dialyzing. The second time the dialysis is continued until no more chlorine is given off. The product is dehydrated with increasing concentration of alcohol, with absolute alcohol, and absolute ether, and is dried in a vacuum over sulfuric acid, then is dried in a hot water oven, and finally in a hot air oven at 105°C. for an hour. The needles should not remain too long in water during the first part of the purification as they are likely to become denatured and cannot then be redissolved in salt solution.

*Concanavalin A.*—The press-cake from which urease and concanavalin B were prepared is washed once with 30 per cent alcohol and pressed out again in the tincture press. This serves to remove sediment that is difficult to filter later on. The press-cake is now mixed up with 2 liters of 2 per cent aqueous sodium chloride solution and again pressed out. The extract is mixed with some toluene and filtered into a dialyzing membrane and the material is dialyzed against 0.001 N acetic acid until the globulins have precipitated completely. We have found that our dialysis parchment contains a considerable amount of a soluble carbohydrate and have removed this by washing the parchment many times in warm water before use. The precipitate by dialysis consists of canavalin, concanavalin A, and a small amount of concanavalin B. The precipitate is centrifuged off and washed with water. The canavalin and concanavalin B are now dissolved by the addition of 10 per cent salt solution. Concanavalin A is not dissolved

and is centrifuged off and washed several times with 10 per cent salt solution and once with distilled water, then it is centrifuged down as hard as possible. The bisphenoid crystals are now dissolved by stirring each 125 cc. of moist precipitate with 100 gm. of solid sucrose and 60 cc. of neutral phosphate solution, containing 6.8 per cent $K_2HPO_4$ and 4.25 per cent $KH_2PO_4$. The solution is treated with neutral phosphate solution, alcohol to 30 per cent, and solid sodium chloride to precipitate the pentosan. After standing for 24 to 48 hours it is filtered and the filtrate is dialyzed until practically free from salt. The crystals are washed until salt-free, dehydrated with alcohol and ether, and dried as described under concanavalin B.

*Canavalin.*—The salt solution of canavalin and concanavalin B, from which concanavalin A has been separated, is dialyzed until the globulins have precipitated. The precipitate is centrifuged off and washed with distilled water and dissolved in 2 per cent sodium chloride. The needles will not dissolve and are centrifuged off and purified as described under concanavalin B. The solution of canavalin is filtered and dialyzed until precipitated, again dissolved in salt solution, and treated with excess of phosphate and sodium chloride and alcohol to 30 per cent. After 24 to 48 hours it filtered clear and again dialyzed. This time the precipitated globulin is centrifuged off and washed with 30 per cent alcohol. It is then dehydrated with alcohol, washed with ether, and dried as has been already described. In drying large masses of protein in a desiccator under vacuum it is important not to use very much suction at first as the material is certain to shoot out of the centrifuge tubes with considerable force.



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## FAT-SOLUBLE VITAMINS.

### XXIII. THE INDUCTION OF GROWTH-PROMOTING AND CALCIFYING PROPERTIES IN FATS AND THEIR UNSAPONIFIABLE CONSTITUENTS BY EXPOSURE TO LIGHT.*

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With the Cooperation of Mariana T. Nelson, C. A. Hoppert, and Blanche M. Riising.

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(Received for publication, April 24, 1925.)

In our report of the discovery of induction of growth-promoting and calcifying properties in various materials by exposure to light (1) we inserted brief mention of the fact that growth-promoting properties had also been imparted to fats. At that time, the rats used in these experiments had been on the fat diet for only a few weeks and it was not considered advisable to kill them immediately for data on the deposition of calcium salts. In spite of the absence of this information, we were nevertheless convinced, in the light of other experiments (2, 3, 4, 5), that in all the instances where a growth-promoting property had been imparted, we were dealing with the production of an antirachitic activation. We have previously expressed our conviction that the antirachitic factor functions, not only in the retention of calcium in rickets, but also in the adult, in which rickets is never observed (6). In the sense in which we use the term, the antirachitic factor represents specifically the organic agent which promotes normal calcium anabolism. It may cure rickets, it may promote growth, or it may simply prevent excessive loss of lime from the body as revealed by metabolism experiments on animals which show no gross abnormality. The specific capacity in which it functions in an animal depends upon the condition of the animal, both with respect to age and nutrition, and upon the composition of the ration furnished. These relations must of necessity be rather intricate and can hardly

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be discussed in this paper from the different angles in which we have come to appreciate them. For the present we must confine our treatment to the antirachitic activation of various fats and their constituents. We have studied this matter in some detail, using growth, increased calcium content of bone, and "line tests" as criteria. We have paid particular attention to the mechanism of the reaction with respect to time, nature of light, permanency of the activation, kind of fat, constituents of fat which can take the activation, and the rôle of sterols. The subject is so immense in its potentialities that we consider our treatment far from satisfactory. We are adding to our information as rapidly as facilities permit.

## EXPERIMENTAL.

### *Part I. Experiments with Irradiated Fat on Growing Rats.*

We have already stated that at the time of our discovery of the activation of fats by exposure to light we used olive oil and lard for the experiments (1), but we did not mention the fact that we compared them with cod liver oil in the effect produced. This comparison was made on the assumption, that if cod liver oil owed its activity to light, it should not undergo any deterioration on further exposure. Obviously this disregarded many factors possibly involved, but at any rate it furnished us a tangible means of approach to the problem.

As before, we used a quartz mercury vapor lamp of the Cooper-Hewitt BY type run at 40 volts and 4.5 amperes as our source of light. The fats were exposed to its radiations at a distance of 22 inches in 100 cc. quantities contained in a glass dish measuring 8 by 10 inches. They were stirred every 5 to 10 minutes. The lard and olive oil were irradiated 30 minutes, but the cod liver oil was irradiated 17 hours.

The rats used were our usual stock animals reared under our standard conditions designed to produce animals low in their reserve of the antirachitic factor. They were confined in our standard cages provided with false, screen bottoms and were fed a basal diet of purified casein, 18; salt 40, 4 (4); yeast, 8; agar, 2; and dextrinized corn-starch, 68. When the fats were fed, they were mixed in with the above ration, 2 parts for each 98 gm. of the mixture.

It has already been reported that at the end of 3 weeks the rats on the irradiated olive oil weighed from 92 to 98 gm. in contrast with the controls at 67 to 73 gm., and that those on the irradiated lard weighed 92 to 125 gm. compared with their controls at 74 to 94 gm.

Chart I in presenting the completed records of these experiments shows that the difference in weight of the different groups

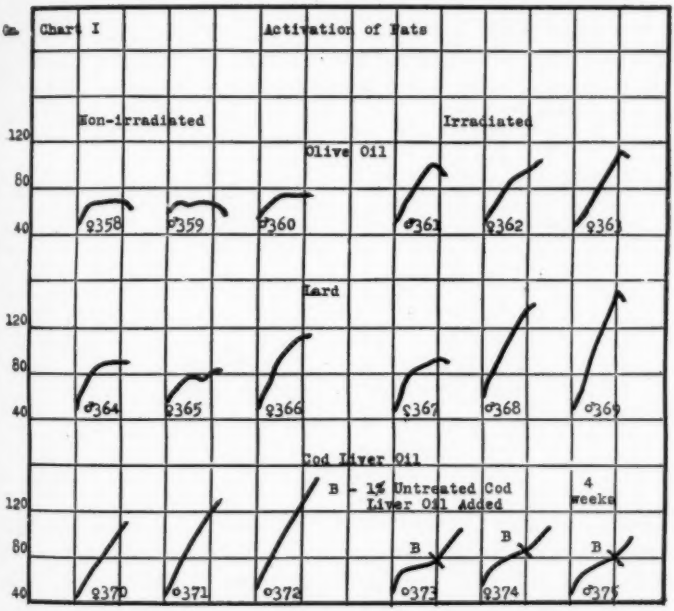


CHART I.

was somewhat increased with prolongation of the experiment. In general both the lard and olive oil rats benefited by irradiation of the fat. One exception, *viz.* Rat 367, was undoubtedly due to a catarrhal condition and therefore merits little consideration.

Contrary to expectations, cod liver oil lost its activity by prolonged irradiation. This apparently was not due to an induced toxicity because the further addition of 1 per cent of untreated oil gave an almost immediate response in growth.

With the termination of the above experiments the animals were killed and the bones removed for ash analyses. These were made on the alcohol-extracted humeri and femora as described in another publication (7). The results are presented only in part (Table I) to conserve space. They show, what we have previously shown to be true for other constituents of the ration, that hand in hand with the stimulation of growth, calcification of the bones is also increased.

That deactivation of the cod liver oil was probably caused by destruction of activated constituents of the oil is supported by the results presented in the first five sets of curves shown in Chart II. In these experiments olive oil was exposed to the ultra-violet rays for respectively 30 minutes, 5 hours, 10 hours, and 17 hours. One

TABLE I.

*Increase in Ash Content of Bones with the Feeding of Irradiated Olive Oil.*

| 2 per cent non-irradiated olive oil. | | | | 2 per cent irradiated olive oil. | | | |
|---|---|---|---|---|---|---|---|
| Rat No. | On ration. | Ash. | | Rat No. | On ration. | Ash. | |
| | | Humerus | Femur. | | | Humerus | Femur. |
| | *days* | *per cent* | *per cent* | | *days* | *per cent* | *per cent* |
| 358 | 35 | 48.8 | 50.9 | 361 | 35 | 53.9 | 52.3 |
| 359 | 35 | 46.4 | 47.1 | 362 | 35 | 56.1 | 55.2 |
| 360 | 35 | 50.0 | 49.2 | 363 | 35 | 58.3 | 56.9 |
| Average..... | | 48.4 | 49.1 | Average..... | | 56.1 | 54.8 |

group served as a control without exposure. The data show that at 30 minutes and even 5 hours exposure, apparently maximum effects of activation comparable to those of our previous trials were secured, but with longer exposure, 10 and 17 hours respectively, such activation had been entirely destroyed.

The last set of curves in Chart II gave us our first data on the permanency of the activation. The test was run on one of our older samples of olive oil, this one in particular having stood in a cork-stoppered Erlenmeyer in a dark cabinet for 5 to 6 weeks before it was used. As seen in the chart it was decidedly growth-promoting. Since these results were obtained we have carried out other tests at intervals of months which will be discussed later in this paper.

Chart III presents further data on the activation of olive oil with rats taken from two different litters. One-half of each litter

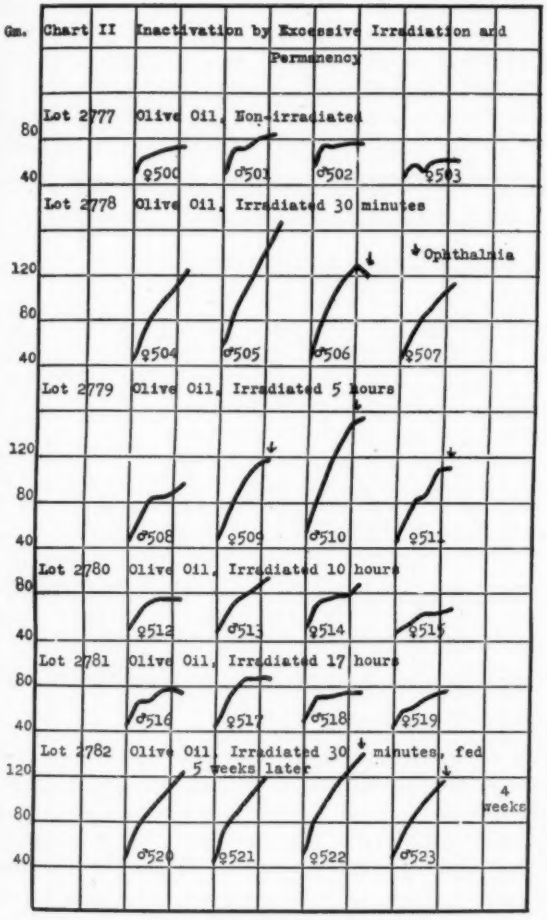


Chart II.

was irradiated and the others were kept as controls. If there remained any doubt in our minds as to the effect of light on the fats or as to the reliability of our technique this dispelled it.

Zucker, Pappenheimer, and Barnett (8), and Steenbock, Jones and Hart (9) showed some time ago that the calcifying properties of cod liver oil were not destroyed by saponification, definitely associating in this way the antirachitic property with the unsaponifiable constituents of the fat. To see if this was also the case with artificially activated oils we irradiated olive oil as before for 30 minutes and then saponified it by boiling it in 50 gm. quantities with 120 cc. of a 20 per cent solution of KOH in alcohol for

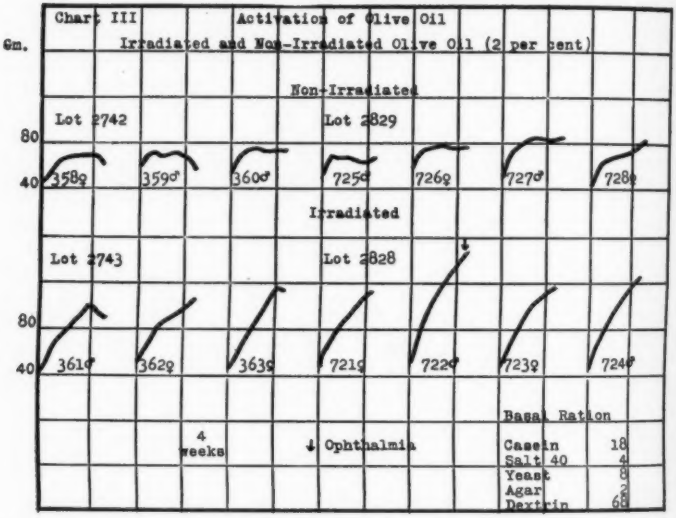


CHART III.

30 minutes. After cooling, the product was diluted with distilled water and shaken out three times with ether. The ether extract was evaporated on the basal ration in a quantity equivalent to 2 per cent of the oil. This was fed to the first group. For comparison there was also prepared a similar product from non-irradiated oil. This was fed to a second group at the same level of intake. A third group received 2 per cent of irradiated oil without any treatment.

Chart IV in the first three sets of curves shows the growth made by the animals on these materials. Irradiated oil and its saponi-

fiable constituents are seen to be fully equivalent to each other, while the unsaponifiable fraction from non-irradiated oil—pre-

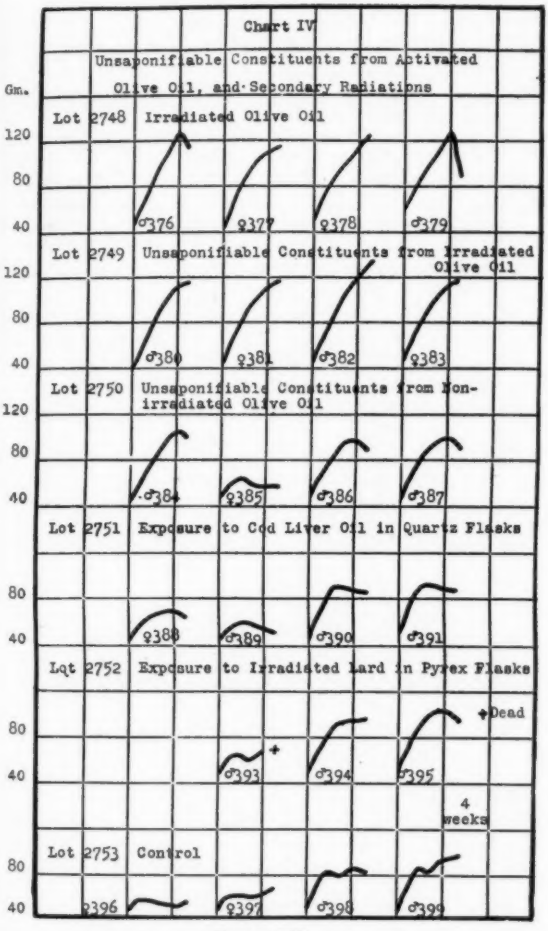


CHART IV.

sented at the bottom of the chart—showed itself to induce little more growth than the basal ration itself.

In Chart IV are also shown two sets of curves, those of Lots 2751 and 2752, which present the results of our first attempts to

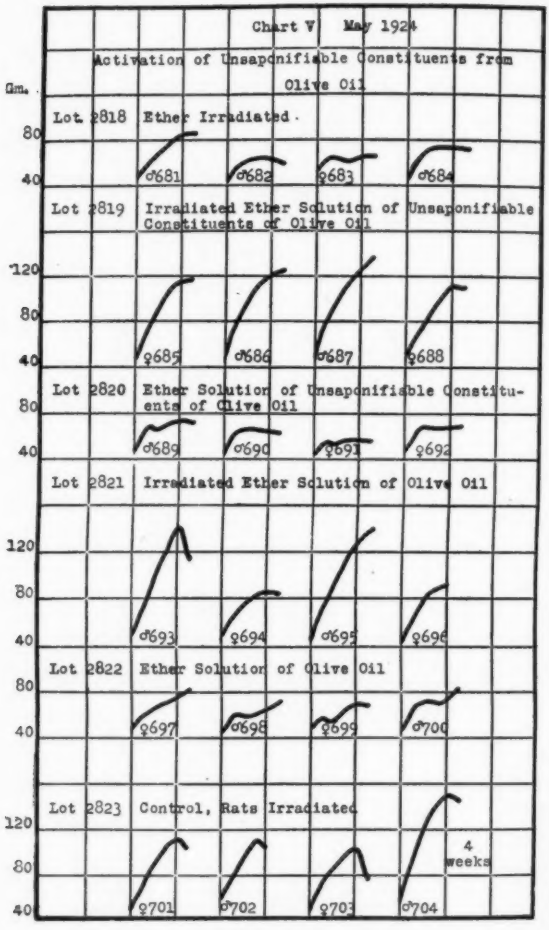


CHART V.

determine the nature of the calcifying and growth-promoting activity of our artificially activated fats and cod liver oil. We

tried to ascertain if these fats give off secondary radiations; in a general way possibly to be compared to a phosphorescence of which we were reminded. We placed two 250 cc. quartz flasks filled with cod liver oil in our rat cages in such a manner that the animals were confined in close proximity to them. As a matter of fact we reduced the size of our cages to one-fourth of their usual dimensions so that the animals were usually in contact with the flasks.

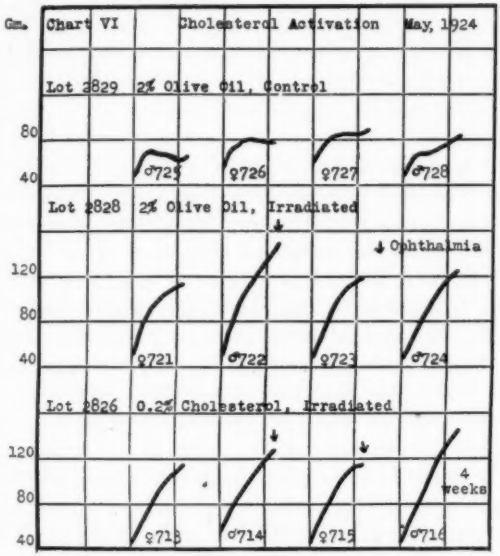


CHART VI.

In another case we took irradiated lard contained in two 200 cc. Pyrex flasks. Needless to say, in all these experiments light other than diffuse daylight and incandescent Mazda lamp light was excluded from the room, for, although these experiments were carried out in March (1924) when solar radiation is not intense no reliance was placed on window glass to filter out the active rays, all windows except those of northern exposure were kept shaded with window curtains. As seen from the chart our results were entirely negative.

Chart V presents still further data on the activation of the unsaponifiable constituents of fats. Here we took the unsaponifiable constituents from olive oil (obtained as before) and irradiated them in ether solution contained in a quartz flask. After irradiation, the solution was evaporated on the ration in such quantity that each 100 gm. received an amount equivalent to 2 gm. of oil. There were also fed portions of the basal ration on which had been evaporated an irradiated ether solution of the unsaponifiable constituents, an irradiated ether solution of olive oil, as well as non-irradiated olive oil and non-irradiated unsaponifiable constituents. In all cases where olive oil or its preparations were fed they were given in such a quantity that 100 gm. of ration received the unsaponifiable constituents from 2 gm. of oil. To further control our experiments, one group of rats on the basal ration was directly irradiated 10 minutes daily 6 days out of the week. As Chart V shows, positive results were obtained only when irradiated unsaponifiable constituents were fed as such or as contained in olive oil, and where the rats were directly irradiated.

With the demonstration that the unsaponifiable constituents of a crude fat could be activated, we next attempted to ascertain if any of the known constituents of this fraction were concerned in this reaction. We had in mind specifically the sterols. In a preliminary way, we dissolved cholesterol prepared from pigs' brain in ether and irradiated it in a quartz flask. After 30 minutes of exposure under the same conditions as the fats, the solution was evaporated on the ration so that 0.2 gm. of cholesterol was added to each 100 gm. of ration. Chart VI shows that this cholesterol was as active as 2 per cent of irradiated olive oil. Previous trials had shown us that non-irradiated cholesterol was entirely inactive, thus furnishing us with the suggestion that at least one sterol was capable of being activated. We speak of it here as a suggestion only because sterols are well known to be purified with difficulty and we had no guarantee that slight amounts of unrecognizable impurities might not have been responsible. Certainly the results of experiments carried out with cholesterol of the degree of purity as purchased on the market cannot be considered final.

*Part II. Experiments with Irradiated and Non-Irradiated Fats on Rachitic Rats.*

During the spring of 1924 when practically all of our experiments were of short duration—6 to 8 weeks or less—we were obliged to breed our rats continuously in order to secure a sufficient number of young. This proved to be a heavy drain on the vitality of our females and made it necessary to secure new breeding stock at an early date. To provide such in excellent condition, we departed from our usual procedure and irradiated all our stock with ultra-violet light and also supplemented their ration with 5 per cent of butter fat and fresh green grass, *ad libitum*. These additions may have been unnecessary; but at all events we were soon well supplied with young. However, we found that our troubles had but just begun, for when we put the young on the basal synthetic ration used in our growth experiments they all grew normally without the addition of any of the antirachitic factor. This compelled us to use, either the line test as developed at the Johns Hopkins Laboratory (10), or else the prevention of rickets, as criteria of the induction of calcifying properties.

Before this time we had had but limited experience with rickets and with the line test merely enough to demonstrate that these methods gave the same results as our growth method; but we had also experienced that while the McCollum Ration 3143 (11) was more satisfactory than the Sherman and Pappenheimer Ration 84 (12), it left much to be desired. In the first place, it did not contain a sufficiency of vitamin A for animals as raised in our stock. Ophthalmia often became incident in our animals before the symptoms of rickets had developed to a sufficient extent to make changing of the ration for an experimental trial permissible. This was true even when we made it a point to use yellow corn which McCollum does not specifically recommend. In the second place, the progress of rickets in our animals was far more variable than could be countenanced with indifference, necessitating most careful control. The variation in the incidence of rickets suggested to us that after rickets had been produced a corresponding variation might also be shown in the response of the animals to the addition of the antirachitic factor. We began to suspect that McCollum's

Diet 3143 carried far more protein than necessary—approximately 33 per cent—and especially too much gelatin. Sometimes our animals developed anorexia—a reaction not unusual with high gelatin diets—and at other times, they had diarrhea.

In the course of these experiments we have accordingly cast about for other more suitable rations. After a large number of trials we found that our Ration 2965 composed of yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1, gave far more consistent results than any other tested. This also had the advantage of greater ease of preparation and reduced cost. Before its general use we also used our Ration 2966 which is the same as Ration 2965 with 5 parts of gelatin substituted for 5 of yellow corn. This was found to possess no superior qualities over Ration 2965. At other times we have used our Ration 3075 carrying 1 part of alfalfa in place of 1 of yellow corn in Ration 2965. This serves to increase the vitamin A content which is sometimes desirable when the experiment is run over a long period of time and severe symptoms of rickets are desired. In this case this amount of alfalfa did not add materially to the amount of the antirachitic factor in the ration, but with 5 parts of alfalfa we found the rickets-producing properties of the ration considerably decreased. It is, however, impossible to make any definite statement in regard to the amount of alfalfa which can be used with safety because it varies in its rickets-producing properties according to the extent it has been previously exposed to light. This we have already reported on for Ration 3143 exposed to ultra-violet light (5), and has also been found by us to be true for clover. 5 per cent of clover cured in the dark has no antirachitic properties, but the same amount cured in the sun may prevent rickets entirely. When completely activated it takes but a few per cent of most naturally occurring foods in a rickets-producing ration to change it completely to one physiologically satisfactory.[1]

In studying the activation of oils by light as determined by the line test, we found it desirable first of all to find out in what time a response to the antirachitic addition was to be expected. For this purpose we used a sample of commercial cod liver oil as the antirachitic agent. Six rats, all from one litter, were kept for

[1] Unpublished data.

35 days on Ration 3143 to develop marked symptoms of rickets. 2 per cent of cod liver oil were then added to the ration and after the 2nd day one animal was killed daily and examined for calcium deposition by silver staining in the metaphyses of the distal ends of the radii and ulnæ. As shown in Table II it was not until the end of the 5th day that a positive line test was obtained. In another trial, with as large an amount of cod liver oil as 10 per cent of the ration, the response was not definitely positive in 2 days. These results, in a general way, correspond to those obtained by McCollum and coworkers (13) who obtained a positive test in 5 days. Evidently, a comparatively low level of antirachitic, over a long period of time, is to be preferred to a high level, over a short period. We have accordingly seen fit to keep our rats at least 7 days and usually 10 to 11 days on the test ration.

TABLE II.

*Time Necessary for Production of Positive Line Test.*

| Lot No. | Rat No. | Basal ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 2837 | 775 | 3143 | 2 per cent cod liver oil. | 2 | 80 | | 5 | Negative. |
| | 776 | | 2 " " " " " | 3 | 79 | | 7 | " |
| | 777 | | 2 " " " " " | 4 | 97 | | 6 | " |
| | 778 | | 2 " " " " " | 5 | 92 | | 5–6 | Positive. |
| | 779 | | 2 " " " " " | 6 | 74 | | 5–6 | " |
| | 780 | | 2 " " " " " | 7 | 75 | | 5 | " |
| 2904 | 1080 | 3143 | 10 " " " " " | 2 | 44 | 44 | 4.5 | Incipient(?). |
| | 1081 | | 10 " " " " " | 2 | 47 | 45 | 4 | " |
| | 1082 | | 10 " " " " " | 2 | 70 | 67 | 7 | " |

It was also necessary for us to know at what level irradiated oils could be fed to best advantage. Table III shows what results are to be expected when irradiated olive oil is fed at lower levels than 2 per cent. It is seen that in a period of 9 days 0.1 per cent was entirely inactive while 0.2 per cent gave fairly positive results. This corresponds with the activity of many samples of cod liver oil as obtained on the market. Such activity is not, however, to be considered a maximum attainable with artificially

TABLE III.

*Amounts of Irradiated Olive Oil Necessary for Positive Line Test.*

| Lot No. | Rat No | Basal ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 2917 | 1146 | 3143 | None. | 9 | 85 | 87 | 7–8 | Negative. |
| | 1147 | | 0.1 per cent irradiated olive oil BI. | 9 | 82 | 84 | 8 | " |
| | 1148 | | 0.1 " " " " " " | 9 | 85 | 87 | 8 | " |
| 2918 | 1149 | | None. | 9 | 84 | 81 | 78 | |
| | 1150 | | 0.2 per cent irradiated olive oil BI. | 9 | 77 | 87 | 7–8 | Questionable. |
| | 1151 | | 0.2 " " " " " " | 9 | 64 | 64 | 6 | Positive. |
| | 1152 | | 0.2 " " " " " " | 9 | 70 | 73 | 7 | " |
| 2919 | 1153 | | None. | 9 | 72 | 78 | 6–7 | Negative. |
| | 1154 | | 0.5 per cent irradiated olive oil BI. | 9 | 72 | 78 | 6 | Positive. |
| | 1155 | | 0.5 " " " " " " | 9 | 65 | 70 | 7 | " |
| | 1156 | | 0.5 " " " " " " | 9 | 62 | 67 | 6 | " |
| 2920 | 1157 | | None. | 9 | 77 | 80 | 8 | Negative. |
| | 1158 | | 1.0 per cent irradiated olive oil BI. | 9 | 67 | 70 | 8 | Positive, broad line. |
| | 1159 | | 1.0 " " " " " " | 9 | 62 | 65 | 6 | " " " |
| 2921 | 1160 | | None. | 9 | 67 | 74 | 8 | Negative. |
| | 1161 | | 2.0 per cent irradiated olive oil BI. | 9 | 70 | 70 | 6 | Practically complete healing. |
| | 1162 | | 2.0 " " " " " " | 9 | 75 | 76 | 6 | " " " |

TABLE IV.

*Permanency of Activity Imparted to Olive Oil by Irradiation.*

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1162 | | 2.0 " " " " " " | 9 | 75 | 76 | 6 | | |

TABLE IV.

*Permanency of Activity Imparted to Olive Oil by Irradiation.*

| Lot No. | Rat No. | Basal ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 3069 | 1838 | 2965 | 0.5 per cent irradiated olive oil BI kept in bottle in dark 8 mos. | 11 | 84 | 86 | 7 | Positive. |
| | 1839 | | 1.0 per cent irradiated olive oil BI kept in bottle in dark 8 mos. | 11 | 92 | 95 | 8–9 | " |
| | 1840 | | 2.0 per cent irradiated olive oil BI kept in bottle in dark 8 mos. | 11 | 94 | 80 | 7 | " |
| 3161 | 2326 | | 0.1 per cent irradiated olive oil BI kept in bottle in dark 10 mos. | 11 | 73 | 77 | 7 | Traces. |
| | 2327 | | 0.2 per cent irradiated olive oil BI kept in bottle in dark 10 mos. | 11 | 84 | 92 | 7 | " |
| | 2328 | | 0.5 per cent irradiated olive oil BI kept in bottle in dark 10 mos. | 11 | 77 | 82 | 6–7 | Positive. |
| | 2329 | | 1.0 per cent irradiated olive oil BI kept in bottle in dark 10 mos. | 11 | 79 | 81 | 7 | Very broad. |
| | 2330 | | 2.0 per cent irradiated olive oil BI kept in bottle in dark 10 mos. | 11 | 79 | 74 | 6 | Almost healed. |
| 3160 | 2320 | | 0.1 per cent irradiated olive oil D II kept exposed to air and light 9 mos. | 11 | 104 | 120 | 8 | Negative. |
| | 2321 | | 0.2 per cent irradiated olive oil D II kept exposed to air and light 9 mos. | 11 | 119 | 138 | 8 | " |
| | 2322 | | 0.5 per cent irradiated olive oil D II kept exposed to air and light 9 mos. | 11 | 95 | 105 | 7–8 | " |
| | 2323 | | 1.0 per cent irradiated olive oil D II kept exposed to air and light 9 mos. | 11 | 114 | 119 | 7 | " |
| | 2324 | | 2.0 per cent irradiated olive oil D II kept exposed to air and light 9 mos. | 11 | 85 | 94 | 6–7 | " |

activated preparations. It is also well known that cod liver oils differ widely in their potency, depending upon their source and method of preparation.

In December we (14) reported that an olive oil activated in April, 1924, had not depreciated in antirachitic potency after 8 months standing in the dark in a stoppered bottle. Hess and

TABLE V.

*Optimum Time of Exposure for Antirachitic Activation of Olive Oil Fed at a 2 Per Cent Level.*

| Lot No. | Rat No. | Basal ration No. | Time of exposure. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | *min.* | *days* | *gm.* | *gm.* | *gm.* | |
| 2901 | 1061 | 3143 | 0 | 7 | 65 | 67 | 6 | Negative. |
| | 1062 | | 5 | 7 | 65 | 64 | 5 | Deposit in center. |
| | 1063 | | 10 | 7 | 65 | 65 | 5 | Narrow line. |
| | 1064 | | 15 | 7 | 60 | 62 | 5 | Heavy line. |
| | 1065 | | 20 | 7 | 62 | 58 | 5 | Very heavy line. |
| | 1066 | | 30 | 7 | 62 | 60 | 5 | " " " |
| 2902 | 1067 | | 0 | 7 | 68 | 77 | 6 | Negative. |
| | 1068 | | 5 | 7 | 67 | 67 | 7 | Fairly heavy line. |
| | 1069 | | 5 | 7 | 67 | 67 | 5 | " " " |
| | 1070 | | 10 | 7 | 75 | 74 | 7 | Very heavy line. |
| | 1071 | | 10 | 7 | 77 | 72 | 5 | " " " |
| | 1072 | | 15 | 7 | 67 | 64 | 6 | Heavy line. |
| 2903 | 1073 | | 15 | 7 | 74 | 70 | 5 | " " |
| | 1074 | | 0 | 7 | 75 | 77 | 5 | Negative. |
| | 1075 | | 20 | 7 | 72 | 72 | 7 | Very heavy line. |
| | 1076 | | 20 | 7 | 69 | 67 | 5 | Heavy line. |
| | 1077 | | 30 | 7 | 57 | 52 | 5 | Very heavy line. |
| | 1078 | | 30 | 7 | 54 | 54 | 6 | Heavy line. |

Weinstock (15) at the same time reported a persistence of activity for a 6 month period. We are now able to report another test after a 10 month interval which showed results substantially the same as before (Table IV). These results are not to be taken as indicating that the artificially induced activity cannot be destroyed readily under certain conditions. We have found one sample of olive oil, activated in May, 1924, and kept in our office till February, 1925, in a beaker covered with a watch-glass, entirely

inactive when fed at a level of 2 per cent. It had also lost its yellow color completely even though exposed to only diffuse daylight.

As to time of exposure with our equipment, we have selected 30 minutes, though at times we have used 15 minutes. This apparently gave optimum results, as shown in Table V, with

TABLE VI.

*Antirachitic Activation of Olive Oil with the Carbon Arc.*

| Lot No. | Rat No. | Ration No. | Time of exposure of dietary addition of 2 per cent olive oil. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | *min.* | *days* | *gm.* | *gm.* | *gm.* | |
| 2983 | 1442 | 2966 | 0 | 10 | 94 | 102 | 9 | Negative. |
| | 1443 | | 5 | 10 | 125 | 134 | 11 | " |
| | 1444 | | 5 | 10 | 112 | 120 | 11 | " |
| | 1445 | | 15 | 10 | 90 | 91 | 8 | " |
| | 1446 | | 15 | 10 | 100 | 97 | 9 | " |
| | 1447 | | 30 | 10 | 87 | 84 | 6–7 | Not continuous. |
| 2984 | 1448 | | 30 | 10 | 105 | 94 | 7–8 | " " |
| | | | *hrs.* | | | | | |
| | 1449 | | 1 | 10 | 84 | 82 | 5–6 | Broad. |
| | 1450 | | 0 | 10 | 87 | 86 | 5 | Negative. |
| | 1451 | | 2 | 10 | 102 | 100 | 6 | Broad. |
| | 1452 | | 2 | 10 | 90 | 88 | 6 | " |
| | 1453 | | 1 | 10 | 90 | 89 | 6–7 | " |
| 2985 | 1454 | | 5 | 10 | 84 | 74 | 5 | Narrow but definite. |
| | 1455 | | 5 | 10 | 89 | 84 | 6 | " " " |
| | 1456 | | 10 | 10 | 100 | 98 | 7 | " " " |
| | 1457 | | 10 | 10 | 85 | 77 | 7 | " " " |
| | 1458 | | 0 | 10 | 97 | 97 | 7 | Negative. |
| 2986 | 1459 | | 17 | 10 | 109 | 117 | 9 | Positive(?). |
| | 1460 | | 17 | 10 | 97 | 94 | 7 | " |
| | 1461 | | 0 | 10 | 99 | 109 | 7–8 | Negative. |

olive oil exposed in a thin layer. In the experiments there reported 25 cc. of olive oil were measured out in a Petri dish 9.7 cm. in diameter, giving a depth of oil from 3 to 4 mm. This was exposed at a distance of 2 feet from our quartz Cooper-Hewitt mercury arc run at 50 volts and 4 amperes. This strength of

current with our apparatus gave a light of low intensity and as the burner had been in daily use for some months its original efficiency was no doubt much reduced.

Other sources of ultra-violet can also be used as shown by the results of Table VI. Here we used an open carbon arc run at 50 to 60 volts at a distance of 20 inches from the oil. The oil was contained in a Petri dish of the same size as before—25 cc. to a dish. To prevent excessive heating and contamination of oil with flakes of carbon, we used an air current from an electric fan. 5 and 15 minute exposures gave entirely negative results and even 30 minutes gave only a faint deposit of calcium salts. Though the data of carbon arc and quartz mercury vapor lamp illuminations are not absolutely comparable they suggest that the carbon arc is not as efficient in effecting the antirachitic activation as the mercury arc. More data are required before a final statement can be made, but it can be accepted that varied sources of ultra-violet light can be used. We have also obtained activation with the iron arc and by exposure to sunlight.

Hess and Weinstock (16) have reported very interesting results, using Corning glass filters in attempting to determine to what particular rays the antirachitic action of light on rats might be due. They found that "in order to be of value in rickets, ultra violet waves must have a wave length not longer than 303 or possibly 313 millimicrons."

We have also used Corning filters,[2] in part the same as those used by Hess and Weinstock, to determine in a preliminary way what rays are responsible for the antirachitic activation of foods. For this purpose we measured 2 cc. of olive oil in inverted porcelain crucible covers about 4 cm. in diameter; covered them with 2 inch squares of the various filters and exposed them simultaneously at a distance of about 20 inches from our mercury arc. The period of exposure was 1 hour. Table VII presents the data obtained. In the first place it shows that Pyrex if sufficiently thin—approximately 1.5 mm.—will transmit the activating rays of the intensity used; and that window glass and plate glass and the Corning glass

[2] The transmission spectra of these filters for ultra-violet light have been published in Technologic Papers 119 and 148 of the U. S. Bureau of Standards.

TABLE VII.

*Antirachitic Effect of Mercury Arc with 2 Per Cent Olive Oil Irradiated 1 Hour Through Filters.*

| Lot No. | Rat No. | Ration No. | Filter. | Filter thickness. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *mm.* | *days* | *gm.* | *gm.* | *gm.* | |
| 2998 | 1521 | 2966 | Pyrex. | 6.00 | 10 | 93 | 100 | 8 | Negative. |
| | 1522 | | " | 6.00 | 10 | 84 | 85 | 7–8 | " |
| | 1523 | | " | 5.02 | 10 | 90 | 102 | 7 | " |
| | 1524 | | " | 5.02 | 10 | 95 | 107 | 7 | " |
| | 1525 | | " | 4.00 | 10 | 85 | 92 | 7 | " |
| 3001 | 1538 | | " | 4.00 | 10 | 80 | 87 | 7–8 | " |
| | 1539 | | " | 3.00 | 10 | 88 | 91 | 6–8 | " |
| | 1540 | | " | 3.00 | 10 | 94 | 104 | 7–8 | " |
| | 1541 | | " | 2.50 | 10 | 95 | 107 | 7 | " |
| | 1542 | | " | 2.50 | 10 | 77 | 81 | 6–8 | Broken line. |
| 3002 | 1543 | | " | 2.00 | 10 | 98 | 103 | 6–8 | Negative. |
| | 1544 | | " | 2.00 | 10 | 100 | 102 | 7 | " |
| | 1545 | | " | 1.49 | 10 | 88 | 88 | 6 | Broken line. |
| | 1546 | | " | 1.49 | 10 | 80 | 82 | 6–7 | " " |
| | 1547 | | " | 0.99 | 10 | 90 | 96 | 6–7 | " " |
| 3003 | 1548 | | " | 0.99 | 10 | 110 | 115 | 8 | Continuous line. |
| | 1549 | | " | 0.75 | 10 | 110 | 109 | 6–8 | Broad line. |
| | 1550 | | " | 0.75 | 10 | 100 | 98 | 8 | " " |
| | 1551 | | Window. | 2.92 | 10 | 90 | 88 | 6–7 | Negative. |
| | 1552 | | " | 2.92 | 10 | 112 | 108 | 6 | " |
| 3005 | 1559 | | " | 1.01 | 10 | 107 | 119 | 9–10 | " |
| | 1560 | | " | 1.01 | 10 | 105 | 107 | 7 | " |
| | 1561 | | Plate. | 4.15 | 10 | 120 | 140 | 9–10 | " |
| | 1562 | | " | 4.15 | 10 | 114 | 127 | 10 | " |
| | 1563 | | " | 2.78 | 10 | 107 | 112 | 8 | " |
| 3006 | 1564 | | " | 2.78 | 10 | 114 | 125 | 10 | " |
| | 1565 | | G-586-A | 3.55 | 10 | 97 | 104 | 6 | " |
| | 1566 | | " | 3.55 | 10 | 95 | 98 | 6 | " |
| | 1567 | | G-38-H | 3.43 | 10 | 107 | 112 | 7 | " |
| | 1568 | | " | 3.43 | 10 | 95 | 96 | 7 | " |
| 3007 | 1569 | | G-385-DP | 3.42 | 10 | 90 | 95 | 7–8 | " |
| | 1570 | | " | 3.42 | 10 | 97 | 104 | 7–8 | " |
| | 1571 | | None. | | 10 | 90 | 89 | 6–7 | Almost complete healing. |
| | 1572 | | " | | 10 | 92 | 92 | 8 | " " |

TABLE VII—*Concluded.*

| Lot No. | Rat No. | Ration No. | Filter. | Filter thickness. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *mm.* | *days* | *gm.* | *gm.* | *gm.* | |
| 3107 | 2027 | 2965 | G-586-A | 3.55 | 10 | 103 | 107 | 7–8 | Negative. |
| | 2028 | | " | 3.55 | 10 | 92 | 90 | 5–6 | " * |
| | 2029 | | " | 3.55 | 10 | 113 | 118 | 8 | " † |
| | 2030 | | " | 3.55 | 10 | 95 | 95 | 5–6 | " † |
| 3108 | 2033 | | Water. | 140 | 10 | 103 | 101 | 7–8 | Continuous line.‡ |
| | 2034 | | " | 140 | 10 | 97 | 93 | 7 | " " ‡ |
| | 2035 | | " | 25 | 10 | 98 | 86 | 6 | " " ‡ |
| | 2036 | | " | 25 | 10 | 130 | 125 | 8–9 | " " |

* Irradiated 4 hours.
† Irradiated 8 hours.
‡ Irradiated 5 hours.

filters G-385-DP, a colorless glass, G-38-H no viol C, a yellow glass, and G-586-A, a purple glass, are impermeable. Pyrex, as is well known, is more transparent to the violet end of the spectrum; and in harmony with this we find that while 1.5 mm. of Pyrex allowed activation to take place, 1.01 mm. of window glass did not.

Inspection of spectrograms taken by Dr. H. P. Gage of the Corning Glass Works showed that the window glass filter transmitted light of even shorter wave-lengths than 313 millimicrons while Pyrex of 1.5 mm. transmitted down to 297 and less. This locates the activating light approximately in the region of the spectrum of no greater wave-length than that spoken of by Hess when using light on the animals directly, but it is to be emphasized that quantitative as well as qualitative factors are involved and absolute conclusions are impossible. In the long time exposures of our olive oil considerable energy in the form of light of shorter wave-lengths than that recorded in the spectrograms may have penetrated the filter and affected the activation. Results obtained with filters in this manner are therefore far from definite. Experiments are now in progress in cooperation with Dr. Farrington Daniels in which we are using monochromatic light on a quantitative basis.

We were surprised at the result obtained with the purple glass

filter, G-586-A—which was negative—not because of its transmission spectrum but because of results published by Hess and Weinstock (16). They made a special point of the fact that, while filter G-586-A transmitted rays which possessed antirachitic properties, another filter, G-86-B, a neutral glass, did not. These filters both transmitted approximately the same rays of the short end of the spectrum, but differed essentially beyond 405 millimicrons. They were led to suggest a neutralizing action of light of one wave-length upon another. We have not been able to confirm their results in this particular, because, not only have we found that an 8 hour exposure under G-586-A is without effect in activating olive oil but a 2 hour daily exposure of rats for 10 days also failed to cure rickets as Hess and Weinstock reported. These results on rats were obtained in duplicate with two different filters, using a new lamp in one case at a distance of 12 inches. Since we obtained these results, Hess and Weinstock (17) also found that filter G-586-A did not allow the passage of light to activate oils, but as far as we know they have not yet reported further results on rats directly.

Knowledge of the transmissibility of various filters for the antirachitic rays is of great importance because of the limited occurrence of antirachitic foods in nature. No doubt, solar radiations of the required wave-lengths are in general able to produce only a superficial effect due to their limited ability to penetrate most materials occurring in nature. Among these, water (18) is a well known exception. That the specific rays of the ultra-violet concerned in antirachitic activation are able to penetrate water, we determined in the following manner. We filled a 250 cc. quartz Florence flask half full of distilled water and, after adding 25 cc. of olive oil and stoppering, inverted it and weighted it down in a glass battery jar almost filled with water. The whole was then exposed to the radiations from a quartz mercury vapor lamp from above for 5 hours. In one case the exposure was made under 25 mm. of water and in another case under 140 mm.

Table VII shows that the olive oil was activated in both cases. Our experiments have not yet determined through what depth of water the rays of the intensity used by us will penetrate, but they suggest how it is possible for cod and other fish to have activated oils stored in their body. Undoubtedly they obtain the active

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TABLE VIII.

*Antirachitic Activation of Fresh Fats as Compared with Old Fats.*

| Lot No. | Rat No. | Ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 2774 | 482 | 3143 | 2 per cent untreated cocoanut oil A — 7 yrs. old. | 6 | 130 | 125 | 8 | Negative. |
| | 483 | | 2 " " " " " " — 7 " " | 6 | 94 | 97 | 6–7 | " |
| | 484 | | 2 " " " " " " — 7 " " | 6 | 135 | 129 | 7 | " |
| | 485 | | 2 " " irradiated " " " — 7 " " | 6 | 100 | 100 | 6 | " |
| | 486 | | 2 " " " " " " — 7 " " | 6 | 115 | 117 | 6 | " |
| | 487 | | 2 " " " " " " — 7 " " | 6 | 84 | 82 | 4–5 | " |
| 2840 | 781 | | 2 " " untreated " " — 2 yrs. old. | 8 | 77 | 77 | 5 | " |
| | 782 | | 2 " " " " " — 2 " " | 8 | 75 | 75 | 6 | " |
| | 783 | | 2 " " irradiated " " — 2 " " | 7 | 72 | 65 | 5 | Positive. |
| | 784 | | 2 " " " " " — 2 " " | 8 | 70 | 65 | 5 | " |
| | 785 | | 2 " " " " " — 2 " " | 8 | 72 | 62 | 4–5 | " |
| 2863 | 885 | | 2 " " untreated fresh cocoanut oil. | 7 | 82 | 87 | 6 | Negative. |
| | 886 | | 2 " " " " " " | 10 | 87 | 82 | 6 | " |
| | 887 | | 2 " " irradiated " " " | 7 | 77 | 78 | 4 | Positive. |
| | 888 | | 2 " " " " " " | 10 | 88 | 87 | 5 | " |
| 3162 | 2331 | | 5 " " untreated " " " | 11 | 89 | 90 | 6 | Negative. |
| | 2332 | | 5 " " " " " " | 11 | 104 | 108 | 6 | " |
| | 2333 | | 10 " " " " " " | 11 | 118 | 112 | 5 | " |
| | 2334 | | 10 " " " " " " | 11 | 112 | 115 | 6 | " |
| | 2335 | | 20 " " " " " " | 11 | 125 | 128 | 6 | " |
| | 2336 | | 20 " " " " " " | 11 | 104 | 102 | 6 | Few specks. |
| 3062 | 1791 | 2965 | 2 " " fresh corn oil — Mazola. | 10 | 82 | 85 | 7 | Negative. |
| | 1792 | | 2 " " " " " " | 10 | 80 | 88 | 7 | " |
| | 1793 | | 2 " " irradiated fresh corn oil— Mazola. | 10 | 95 | 95 | 8 | Positive. |
| | 1794 | | 2 " " " " " " | 10 | 105 | 98 | 7–8 | " |
| 3063 | 1799 | | 2 per cent untreated corn oil — 10 yrs. old. | 10 | 87 | 87 | 7 | Negative. |
| | 1800 | | 2 " " " " " — 10 " " | 10 | 78 | 80 | 6–7 | " |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 3062 | 1791 | 2965 | 2 " " fresh corn oil — Mazola. | 10 | 82 | 85 | 7 | Negative. |
| | 1792 | | 2 " " " " " — " | 10 | 80 | 88 | 7 | " |
| | 1793 | | 2 " " irradiated fresh corn oil— Mazola. | 10 | 95 | 95 | 8 | Positive. |
| | 1794 | | 2 " " " " " — " | 10 | 105 | 98 | 7–8 | " |
| 3063 | 1799 | | 2 per cent untreated corn oil — 10 yrs. old. | 10 | 87 | 87 | 7 | Negative. |
| | 1800 | | 2 " " " " " — 10 " " | 10 | 78 | 80 | 6–7 | " |
| | 1801 | | 2 " " irradiated " " — 10 " " | 10 | 68 | 73 | 6 | " |
| | 1802 | | 2 " " " " " — 10 " " | 10 | 98 | 102 | 8–9 | " |
| 2838 | 769 | 3143 | 2 " " untreated cottonseed oil — 10 yrs. old. | 11 | 77 | 78 | 6 | " |
| | 770 | | 2 " " " " " — 10 " " | 7 | 70 | 69 | 5 | " |
| | 771 | | 2 " " " " " — 10 " " | 17 | 90 | 92 | 4–5 | " |
| | 772 | | 2 " " irradiated " " — 10 " " | 7 | 99 | 100 | 7–8 | " |
| | 773 | | 2 " " " " " — 10 " " | 11 | 87 | 90 | 6 | " |
| | 774 | | 2 " " " " " — 10 " " | 17 | 79 | 74 | 4–5 | " |
| 2997 | 1517 | 2966 | 2 " " " fresh cottonseed oil — Wesson. | 10 | 78 | 78 | 6 | Positive. |
| | 1518 | | 2 " " " " " " — " | 10 | 85 | 85 | 7 | " |
| | 1519 | | 2 " " untreated " " " — " | 10 | 110 | 119 | 8 | Negative. |
| | 1520 | | 2 " " " " " " — " | 10 | 93 | 100 | 8 | " |
| 2775 | 488 | 3143 | 2 " " " peanut oil — 6 yrs. old. | 6 | 93 | 92 | 3–4 | " |
| | 489 | | 2 " " " " " — 6 " " | 6 | 79 | 72 | 5 | " |
| | 490 | | 2 " " " " " — 6 " " | 6 | 102 | 95 | 4–5 | " |
| | 491 | | 2 " " irradiated " " — 6 " " | 6 | 100 | 100 | 5 | " |
| | 492 | | 2 " " " " " — 6 " " | 6 | 124 | 119 | 8 | Faint line. |
| | 493 | | 2 " " " " " — 6 " " | 6 | 101 | 99 | 6 | Negative. |
| 3149 | 2255 | 2965 | 2 " " untreated oleo oil — 6 yrs. old. | 11 | 112 | 117 | 5 | " |
| | 2256 | | 2 " " " " " — 6 " " | 11 | 99 | 107 | 6–7 | " |
| | 2257 | | 2 " " irradiated " " — 6 " " | 11 | 102 | 112 | 7 | " |
| | 2258 | | 2 " " " " " — 6 " " | 11 | 89 | 92 | 6 | " |
| 3148 | 2252 | | 10 " " butter fat. | 10 | 82 | 86 | 6–7 | " |
| 3130 | 2143 | | 2 " " " " | 11 | 81 | 80 | 4 | " |
| | 2144 | | 2 " " " " irradiated. | 11 | 84 | 83 | 6 | Positive |
| | 2145 | | 2 " " " " " | 11 | 102 | 106 | 6 | " |
| 3106 | 2023 | | 5 " " " " " | 11 | 107 | 112 | 6 | Almost healed. |
| | 2024 | | 10 " " " " " | 11 | 92 | 98 | 6 | " " |

compounds from small fish living in rather shallow waters which in turn consume smaller organisms thoroughly exposed to ultraviolet radiations. This suggestion is not new. Saleeby (19) when advocating greater hygienic utilization of sunlight expressed himself as follows: "Not one word against cod liver oil do I suggest. . . . . The vitamin in it was probably made by sunlight falling on the green plankton in the far waters of the North Atlantic, and thence, via other creatures, reached the cod, and the cod's liver, whence at length it gets into the blood of Sheffield children." Drummond and Watson have also pointed out how growth-promoting properties are synthesized by diatoms grown in the sunlight in synthetic media and how these diatoms ultimately and indirectly provide food for the cod (20).

When attempting to activate other fats than lard and olive oil we have obtained variable results. One of the first oils that we used was a sample of cocoanut oil which had been kept in a mason jar in the laboratory for 7 years. It had been exposed to the diffuse light of the laboratory and was decidedly acid. Table VIII shows that when added to Ration 3143 at a 2 per cent level after irradiation, it gave negative results. Another sample, an unrefined oil kept in the laboratory for 2 years in a tin container, while inactive before irradiation, was readily made antirachitic. A sample of fresh cocoanut oil extracted by pressure from cocoanut meats in the laboratory was found inactive, but could be readily activated. In general with laboratory-prepared cocoanut oil we have not as yet been able to demonstrate antirachitic properties by using the line test when as much as 20 per cent of the ration was fed over a period of 10 days or when as much as 60 per cent of oil equivalent was fed as the isolated unsaponifiable constituents. We are inclined to believe that the reported antirachitic potency of commercial cocoanut oil (13, 21) may have been acquired by the usual practice of drying the copra of commerce in the sun. This remains to be tested out experimentally.

A fresh sample of corn oil gave negative results before irradiation and positive results afterwards as shown in Table VIII. But again with a sample of corn oil approximately 10 years old, negative results were obtained even after 30 minutes irradiation. The same was true of cottonseed oil of approximately the same age. The corn oil had been kept in a tightly stoppered tin container

about one-third full and the cottonseed oil in a loosely stoppered glass bottle. Peanut oil and oleo oils, both 6 years old, kept in tin cans, could not be activated. The fresh oils have not yet been tested.

As the result of the widespread recognition of the nutritive value of milk and its products the idea that milk is a panacea for human and animal ills has gained widespread credence. It is, however, common knowledge in medical circles that neither the consumption of cow's milk nor human milk even in large quantity is necessarily a guarantee against the incidence of rickets. Our data on butter fat (Lots 3148, 3130, and 3106, Table VIII) support other evidence (13) of its inefficiency in this capacity. This obviously is due not so much to a poverty in compounds which can be antirachitically activated as to a lack of radiation. Like whole milk and egg yolk (20) the potency of which we have increased from 15- to 20-fold, it too can have its potency increased.

*Part III. Experiments with Irradiated and Non-Irradiated Unsaponifiable Constituents on Rachitic Rats.*

In Part I we described experiments bearing on the antirachitic activation of the unsaponifiable constituents of olive oil as determined by our growth method. We now wish to present data using the "line test" for confirmation of these results, as well as for a more definite characterization of the nature of the compounds which can be activated.

First of all, we again prepared an ether solution of the unsaponifiable constituents of olive oil. This we administered before and after irradiation to rachitic rats. The ether solution was evaporated on the ration in amounts equivalent to 2 per cent of olive oil and when irradiated was always exposed to our mercury arc for 30 minutes at a distance of approximately 2 feet. Table IX confirms our other work in showing that the irradiated material induced calcium deposition in the rachitic rat while the untreated did not. From these results, it is not, however, to be inferred that the ability to become antirachitically active is a property common to unsaponifiable compounds in general. We shall not treat of this matter in detail at this time, but in a preliminary way we show in Table X how mineral oil (Stanolax), for example, can-

not be affected in this manner. This corresponds with data obtained by Hess and Weinstock (17).

TABLE IX.

*Activation of Unsaponifiable Constituents of Fats.*

| Lot No. | Rat No. | Ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 2854 | 839 | 3143 | Unsaponifiable matter equivalent to 2 per cent olive oil. | 11 | 84 | 92 | 8 | Negative. |
| | 840 | | " " | 11 | 77 | 87 | 8 | " |
| | 841 | | " " | 9 | 85 | 82 | 6 | " |
| | 842 | | Unsaponifiable matter equivalent to 2 per cent olive oil irradiated. | 9 | 82 | 80 | 6 | Positive. |
| | 843 | | " " | 11 | | 72 | 6 | " |
| | 844 | | " " | 11 | 102 | 98 | 6–7 | " |

TABLE X.

*Irradiation of Mineral Oil.*

| Lot No. | Rat No. | Ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 2830 | 731 | 3143 | 0.5 per cent untreated mineral oil (Stanolax). | 26 | 114 | 110 | 6 | Negative. |
| | 733 | | 0.5 per cent irradiated mineral oil (Stanolax). | 26 | 87 | 85 | 6 | " |
| 2817 | 677 | | 2.0 per cent untreated mineral oil (Stanolax). | 26 | 89 | 89 | 5–6 | " |
| | 680 | | 2.0 per cent irradiated mineral oil (Stanolax). | 26 | 97 | 96 | 5–6 | " |

Our earlier experiments of May, 1924, showing that ordinary cholesterol represented one of a group of compounds taking the activation, led us to concentrate our attention on cholesterol. We used cholesterol prepared from pig's brain by cold acetone

extraction after drying with plaster of Paris. Purification of it was effected by saponifying it with potassium hydroxide dissolved in alcohol, then extracting it with ether, and repeatedly crystallizing from alcohol. Samples 1 and 2 were recrystallized eight times from 95 per cent alcohol which gave us a product melting at 147°C. (uncorrected). Irradiation of them was carried out in ether solution in a quartz flask with an exposure of 30 minutes at a distance of 2 feet.

Lots 2967 and 2981 in Table XI show that cholesterol prepared as above was activated to a very high degree. One-hundredth of 1 per cent of irradiated cholesterol in a ration, with the total consumption by the rat of 6 mg., gave a very broad calcium line in the distal ends of its radii and ulnæ in 10 days. These results, however, did not assure us definitely that cholesterol represented the acceptor for the radiant energy because sterols are well known to be purified with difficulty. We accordingly took measures to purify our cholesterol further.

4.93 gm. of cholesterol (already recrystallized eight times and activated by exposure to ultra-violet light) were benzoylated by the method of Dorée and Gardner (22), using redistilled benzoyl chloride and pyridine. After pouring into water, the product was filtered off; boiled up with alcohol; cooled; and filtered. This latter was repeated twice and gave us a preparation of cholesterol benzoate melting at 147°C. (uncorrected), with the characteristic changes which this compound undergoes when exposed to higher temperatures.

When fed to rats it was found inactive (Lot 3004). After saponification by boiling with potassium hydroxide, dissolved in alcohol, then extraction with ether, and recovery of the cholesterol by crystallization from alcohol, a cholesterol was obtained which was also found inactive. Its melting point remained unchanged at 147°C. (uncorrected). Irradiation in ether solution, however, was able to restore its activity.

At present we are unable to explain definitely the negative results obtained with the benzoate as prepared from the active cholesterol, because we do not know whether the benzoate as such or the cholesterol in combination with it is absorbed. It appears, however, that the cholesterol is totally inactivated because mother liquors from the recrystallizations of the benzoate when fed to two rats were also found inactive.

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TABLE XI.

*Antirachitic Activation of Cholesterol.*

| Lot No. | Rat No. | Ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 2967 | 1338 | 3143 | 2 per cent olive oil irradiated. | 10 | 97 | 98 | 6 | Positive. |
| | 1339 | | 0.2 per cent cholesterol. | 10 | 104 | 109 | 7–8 | Negative. |
| | 1340 | | 0.2 " " " | 10 | 118 | 119 | 7–8 | " |
| | 1341 | | 0.2 " " " I irradiated. | 10 | 115 | 107 | 6–7 | Positive. |
| | 1342 | | 0.2 " " " I " | 10 | 120 | 114 | 6 | " |
| | 1343 | | 0.2 " " " I " | 10 | 106 | 95 | 4 | " |
| 2981 | 1429 | 2966 | 0.2 " " " II. | 10 | 84 | 89 | 8 | Negative. |
| | 1430 | | 0.2 " " " II. | 10 | 80 | 87 | 8 | " |
| | 1431 | | 0.2 " " " II irradiated. | 10 | 97 | 88 | 5 | Very broad. |
| | 1432 | | 0.1 " " " II " | 10 | 84 | 76 | 5 | Almost complete healing. |
| | 1433 | | 0.05 " " " II " | 10 | 85 | 77 | 6 | " " " |
| | 1434 | | 0.01 " " " II " | 10 | 77 | 77 | 6 | Very broad. |
| 3004 | 1553 | | 0.2 per cent benzoate of irradiated cholesterol I. | 10 | 92 | 105 | 6–8 | Negative. |
| | 1554 | | " " | 10 | 87 | 93 | 6–8 | " |
| | 1555 | | 0.2 per cent cholesterol III from benzoate of irradiated cholesterol I. | 10 | 90 | 102 | 5–6 | " |
| | 1556 | | " " | 10 | 90 | 96 | 7–8 | " |
| | 1557 | | 0.2 per cent irradiated cholesterol III from benzoate of irradiated cholesterol I. | 10 | 97 | 105 | 7–8 | Almost complete healing. |
| | 1558 | | " " | 10 | 102 | 114 | 8 | " " " |
| 3064 | 1809 | 2965 | Mother liquors from benzoate. | 10 | 112 | 107 | 10–11 | Negative. |
| | 1810 | | " " " " | 10 | 102 | 115 | 9 | " |
| 3059 | 1775 | | 0.1 per cent irradiated cholesterol III-A prepared through acetate from cholesterol III. | 10 | 99 | 107 | 8 | Broad. |
| | 1776 | | " " | 10 | 87 | 90 | 7 | Almost complete healing. |

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The cholesterol obtained from the benzoate was purified still more. We took 1 gm. of it after recrystallization from alcohol and acetylated it by heating with acetic anhydride over a Bunsen burner for 10 minutes. On pouring into water the acetate separated out. It was recrystallized from alcohol, giving 0.925 gm. of product, melting at 113.8°C. (uncorrected). 0.5 gm. of this was saponified, yielding after crystallization from alcohol 0.77 gm. of cholesterol. This cholesterol again gave the same melting point; namely, 147°C. (uncorrected) as before. When irradiated and fed at a level of 0.1 per cent, it gave a very broad line test with Rats 1775 and 1776.

Up to this time we had always irradiated our cholesterol in ether solution to good effect. Table XII shows that it can also be activated in crystal form when exposed under our usual conditions. Apparently the time of exposure can be varied tremendously because after an exposure of 17 hours in place of the usual 30 minutes, it is seen that the activity of the cholesterol had been decreased to only a barely detectable degree. It should be emphasized that we have presented only a part of the data. Actually we have irradiated cholesterol for 1 hour, 2 hours, 5 hours, and 10 hours, as well as at 30 minutes and 17 hours, and each preparation was fed at the various levels ranging from 0.01 to 0.2 per cent. Our results showed no discrepancies in the entire series. In view of the fact that the cholesterol was exposed to the air in an open dish during these irradiations, it indicates a remarkable stability on the part of the cholesterol which stands in rather sharp contrast to what we have observed for olive oil.

Table XIII presents some data that we obtained with phytosterol from corn oils. Here we used the same corn oils on which we have already reported in Part II. From these we prepared phytosterols by saponifying them with sodium hydroxide dissolved in alcohol and then extracting the dried soaps with ether. The resultant ether extract was evaporated to dryness, taken up in hot alcohol, and the phytosterols were crystallized out on cooling. Purification was effected by repeated crystallization from hot alcohol in like manner. Phytosterol I was recrystallized four times; phytosterol II, nine times; and phytosterol III, five times. All were beautifully white crystalline compounds, melting respectively at 137°, 138°, and 138.5°.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3088 | 1775 | 0.1 per cent irradiated cholesterol III-A prepared through acetate from cholesterol III. | 10 | 87 | 90 | 107 | 8 | Broad. Almost complete healing. |
| | 1776 | " " | 10 | 87 | 90 | 107 | 7 | |

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TABLE XII.

*Excessive Irradiation of Cholesterol.*

| Lot No. | Rat No. | Ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 3090 | 1934 | 2965 | 0.01 per cent cholesterol V irradiated 30 min. in ether solution. | 10 | 139 | 145 | 9 | Broad. |
| | 1935 | | 0.05 per cent cholesterol V irradiated 30 min. in ether solution. | 10 | 102 | 105 | 7–8 | Almost completely healed. |
| | 1936 | | 0.1 per cent cholesterol V irradiated 30 min. in ether solution. | 10 | 97 | 102 | 7–8 | " " " |
| | 1937 | | 0.2 per cent cholesterol V irradiated 30 min. in ether solution. | 10 | 95 | 100 | 8 | " " " |
| 3093 | 1954 | | 0.01 per cent cholesterol V irradiated 17 hrs. in ether solution. | 11 | 102 | 107 | 8–9 | Not definitely positive. |
| | 1955 | | 0.05 per cent cholesterol V irradiated 17 hrs. in ether solution. | 11 | 104 | 113 | 7–8 | Positive. |
| | 1956 | | 0.1 per cent cholesterol V irradiated 17 hrs. in ether solution. | 11 | 102 | 100 | 7 | Quite broad. |
| | 1957 | | 0.2 per cent cholesterol V irradiated 17 hrs. in ether solution. | 11 | 107 | 107 | 8 | Very broad. |
| | 1958 | | 0.01 per cent cholesterol V irradiated 30 min. as crystals. | 11 | 82 | 85 | 6–7 | Quite broad. |
| | 1959 | | 0.05 per cent cholesterol V irradiated 30 min. as crystals. | 11 | 77 | 75 | 5–6 | Almost completely healed. |
| 3094 | 1960 | | 0.1 per cent cholesterol V irradiated 30 | 11 | 95 | 99 | 6 | Very broad. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1959 | | 0.05 per cent cholesterol V irradiated 30 min. as crystals. | 11 | 77 | 75 | 5–6 | Almost completely healed. |
| 3094 | 1960 | | 0.1 per cent cholesterol V irradiated 30 min. as crystals. | 11 | 95 | 99 | 6 | Very broad. |
| | 1961 | | 0.2 per cent cholesterol V irradiated 30 min. as crystals. | 11 | 103 | 100 | 6–7 | Almost completely healed. |
| 3096 | 1978 | | 0.01 per cent cholesterol V irradiated 17 hrs. as crystals. | 11 | 85 | 85 | 6–7 | Negative. |
| 3104 | 2007 | | 0.05 per cent cholesterol V irradiated 17 hrs. as crystals. | 10 | 93 | 100 | 9 | Positive. |
| | 2008 | | 0.1 per cent cholesterol V irradiated 17 hrs. as crystals. | 10 | 89 | 90 | 7 | " |
| 3108 | 2031 | | 0.2 per cent cholesterol V irradiated 17 hrs. as crystals. | 10 | 109 | 97 | 5 | " |

TABLE XIII.

*Activation of Phytosterol.*

| Lot No. | Rat No. | Ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 3062 | 1795 | 2965 | 0.175 per cent phytosterol III from fresh corn oil (Mazola). | 10 | 90 | 92 | 7 | Negative. |
| | 1796 | | " " | 10 | 94 | 94 | 7 | " |
| | 1797 | | 0.175 per cent irradiated phytosterol III from fresh corn oil (Mazola). | 10 | 88 | 82 | 6–7 | Positive. |
| | 1798 | | " " | 10 | 79 | 76 | 6–7 | " |
| 3063 | 1803 | | 0.2 per cent phytosterol II from old corn oil. | 10 | 90 | 94 | 7 | Negative. |
| | 1804 | | 0.2 " " " II " " " " | 10 | 135 | 116 | 0–1 | (Positive but caused by fasting.) |
| | 1805 | | 0.2 " " irradiated phytosterol II from old corn oil. | 10 | 82 | 87 | 8–9 | Negative. |
| | 1806 | | " " | 10 | 95 | 102 | 8 | Traces. |
| 3026 | 1645 | 3143 | 0.2 per cent phytosterol I from old corn oil. | 10 | 75 | 88 | 6 | Negative. |
| | 1646 | | 0.2 " " " I " " " " | 10 | 82 | 95 | 7 | " |
| | 1647 | | 0.2 " " irradiated phytosterol I from old corn oil. | 10 | 102 | 116 | 9 | " |
| | 1648 | | " " | 10 | 90 | 96 | 7 | " |
| 3078 | 1883 | 2965 | 0.5 per cent irradiated phytosterol II from old corn oil. | 10 | 80 | 80 | 7 | Narrow line. |
| | 1884 | | " " | 10 | 59 | 65 | 6 | Broad line. |
| | 1885 | | 1.0 per cent irradiated phytosterol II from old corn oil. | 10 | 77 | 75 | 7 | " " |
| | 1886 | | " " | 10 | 87 | 85 | 7 | " " |

The data shown in the table corroborate in a general way with the conclusions that we arrived at from our experiments carried out with the oils themselves with the exception that when one of the phytosterols was fed at a higher level than 0.2 per cent of the ration it gave positive results. Apparently there was still a trace of material in the oil which was capable of being activated. It is not clear from these results whether this inactivation represents an isomerization of the sterol or presence of a contamination. We shall report on that later.

When we first observed that old cocoanut oil could not be activated, we suspected that probably the acidity might be responsible. To determine this, we added 5.44 gm. of oleic acid to 100 cc. of fresh olive oil, which gave it an acidity equal to that of the cocoanut oil, and then irradiated the mixture. We found as shown in Table XIV that this did not interfere with the activating processes. We also dissolved the unsaponifiable constituents of olive oil in old cocoanut oil which could not be activated in such amounts that 2 gm. of cocoanut oil carried the unsaponifiable constituents from 2 gm. of olive oil. As the table shows, when this mixture was irradiated it became antirachitically active. We believe as a result of these experiments that we are justified in assuming that the failure of the activation of the old oils is not due to an interference with the process of activation itself but to some molecular change in the unsaponifiable constituents which renders them incapable of accepting the activation.

In this connection it is interesting to note the results obtained by Fridericia (23) when determining what he believed to be vitamin A. He found that old fats, or oxidized butter fat, when melted with fresh untreated butter fat, destroyed the ability of the latter to maintain growth in rats. As Fridericia makes no mention of the incidence of ophthalmia in his animals it is very probable that his animals ceased to grow because of the destruction of the antirachitic factor rather than of vitamin A. This is all the more probable because 10 per cent of butter fat as used by him in compounding his ration would not supply a great excess of the antirachitic agent. His results from this standpoint can be brought into harmony with our own. Quite recently we had occasion to observe that olive oil activated in the presence of oleic acid had lost its activity after standing in the dark for 4 months. This is in sharp

TABLE XIV.

*Analysis of Failure of Activation of Old Fats.*

| Lot No. | Rat No. | Ration No. | Dietary addition. | On modified ration. | Initial weight. | Final weight. | Daily consumption. | Line test. |
|---|---|---|---|---|---|---|---|---|
| | | | | *days* | *gm.* | *gm.* | *gm.* | |
| 2962 | 1344 | 3143 | 2 per cent irradiated olive oil. | 9 | 115 | 110 | 7–8 | Positive. |
| | 1345 | | 2 " " olive oil acidified with oleic acid. | 9 | 98 | 95 | 6 | Negative. |
| | 1346 | | " " | 9 | 85 | 75 | 3–4 | Positive (animal sick with respiratory trouble). |
| | 1347 | | 2 per cent oil acidified with oleic acid, then irradiated. | 9 | 105 | 108 | 6–7 | Positive. |
| | 1348 | | " " | 9 | 75 | 72 | 5–6 | " |
| | 1349 | | " " | 9 | 110 | 112 | 8 | " |
| 2963 | 1351 | | Irradiated non-saponifiable extract of cocoanut oil A equivalent to 2 per cent of oil | 9 | 97 | 95 | 7 | Negative. |
| | 1352 | | " " | 9 | 87 | 87 | 7 | " |
| 2964 | 1356 | | 2 per cent cocoanut oil A plus non-saponifiable extract of olive oil equivalent to 2 per cent olive oil. | 9 | 117 | 116 | 5–6 | " |
| | 1357 | | " " | 9 | 130 | 131 | 7–8 | " |
| | 1358 | | 2 per cent cocoanut oil A irradiated with non-saponifiable extract of olive oil equivalent to 2 per cent olive oil. | 9 | 105 | 102 | 5–6 | Positive. |
| | 1359 | | " " | 9 | 102 | 96 | 6 | " |
| | 1360 | | " " | 9 | 130 | 126 | 8 | " |

contrast to our observations with unacidified olive oil. Furthermore, activated cholesterol has been observed to lose its activity by evaporation with hydrochloric acid.

SUMMARY.

Evidence of the antirachitic activation of fats is presented in considerable detail using the line test, growth, and increased calcium content of bone, as criteria.

Excessive irradiation of olive oil as well as cod liver oil was found to result in their inactivation. Evidently the compounds activated are rather labile in the presence of other constituents of these fats.

Activated olive oil, kept in a stoppered bottle in the dark, was found unimpaired in activity after 10 months.

As in cod liver oil, the antirachitic potency of irradiated fats lies in their unsaponifiable constituents.

Mineral oil cannot be activated.

A ration composed of yellow corn 76, wheat gluten 20, calcium carbonate 3, and sodium chloride 1, was found more satisfactory for producing rickets than any hitherto reported.

With the various rickets-producing rations used, a test period of 7 to 11 days was found sufficient to produce a positive reaction.

Activation was induced by light produced by the quartz mercury vapor lamp, the open carbon arc, the iron arc, and by sunlight.

Activating rays of the quartz mercury vapor lamp were not able to penetrate the Corning glass filters G-385-DP, G-38-H, G-586-A, window glass, nor plate glass. Thin Pyrex was somewhat permeable.

Water was found to transmit the activating rays, thus demonstrating how cod liver oil may possibly acquire its activity.

Old oils such as cocoanut oil, corn oil, oleo oil, peanut oil, and cottonseed oil, all acid in reaction, could not be activated.

The same was true of the unsaponifiable constituents of old oils, including phytosterols with the exception of one case. In this, the irradiated phytosterol showed some activity when fed at high levels.

Cholesterol, purified by repeated crystallizations from alcohol, and then as the benzoate and acetate, could be activated.

The activity of irradiated cholesterol could not be destroyed by long continued irradiation either in crystal form or in ether solution.

The failure of activation of old oils does not lie in an inhibition of the reaction by compounds formed on standing because mixtures of unsaponifiable constituents of fresh oils with old oils can be activated; furthermore, acidification of fresh fats does not prevent activation.

Further data will be presented later.

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# FAT-SOLUBLE VITAMINS.

## XXIV. THE NON-PRECIPITABILITY OF THE ANTIOPHTHALMIC AND ANTIRACHITIC PROPERTIES FROM COD LIVER OIL BY DIGITONIN.*, †

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The stability of vitamin A and the antirachitic factor to saponification has been demonstrated in this and other laboratories (1–8) and is now a well established fact. Unfortunately the localization of these active principles in the unsaponifiable fraction of fats does not augur well for their isolation because of the paucity of data on the nature of the material of which it is constituted. Nevertheless it is well that such compounds as are known to exist in this lipoidal moiety be studied for possible activity. In this connection the sterols immediately suggest themselves.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

† Since the preparation of this manuscript a lecture by Dr. J. C. Drummond (Drummond, J. C., *Chem. and Ind.*, 1924, xliii, 923) has come into the writers' hands. In this lecture Dr. Drummond presented data on the fat-soluble accessory factors showing that they were not precipitated by digitonin, that they could be distilled, and that they were not destroyed by acetylation, benzoylation, and hydrogenation. Apparently Dr. Drummond's otherwise interesting results with respect to the latter can be placed in the same category as those of Steenbock, Sell, Nelson, and Buell (Steenbock, H., Sell, M. T., Nelson, E. M., and Buell, M. V., *J. Biol. Chem.*, 1921, xlvi, p. xxxii) on the stability of the so called fat-soluble vitamin to acetylation and hydrogenation. At that time we also did not realize that under certain conditions the antirachitic factor was necessary for growth. When using reinitiation of growth as a criterion of activity, it is a matter of stored reserves in the animal and inorganic composition of the ration which determines whether a response is due to presence of vitamin A or the antirachitic factor; under certain conditions it may be both.

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Giving consideration to cod liver oil as a fat rich in antiophthalmic and antirachitic principles, the literature is not void of references to sterols and particularly to cholesterol as the constituent responsible for its therapeutic activity. Rosenbaum (9) found no difference in the sterol content of egg yolk fat, cod liver oil, butter fat, human milk fat, lard, and hazel-nut oil that could be correlated with biological value. Takahashi (10) in reporting his attempts to isolate the active principle (vitamin A) of cod liver oil speaks of active principles and cholesterol, inferring that cholesterol is inert. Zucker, Pappenheimer, and Barnett (6) concluded "that the antirachitic substance of cod liver oil can be demonstrated in the ether soluble unsaponifiable fraction after alkaline hydrolysis. It is not an organic base of the type described as occurring in cod liver oil. It is not cholesterol, but similar to cholesterol in its solubilities. The suggestion is made that it may be a sterol related to cholesterol or a cholesterol derivative." From the preceding references and citation, one would conclude that cholesterol found in active cod liver oil is not one of its active therapeutic constituents.

We sought to gain some information on this matter in 1921 when we were accumulating data on the occurrence of the so called fat-soluble vitamin. In looking for exceptions to our hypothesis (11) that the fat-soluble vitamin might be one of the yellow fat pigments, we fed ox brain and bile from the sheep, pig, and ox,—all comparatively rich in unsaponifiable constituents. Our results were not satisfactory. The biles proved to be toxic and the brains fed at 2 and 5 per cent levels did not promote growth nor entirely prevent ophthalmia.[1] These experiments are not to be compared in accuracy with those carried out at the present time, and altogether too much emphasis was placed upon the absence of growth, which undoubtedly was due to an insufficiency of the antirachitic factor rather than to a lack of vitamin A (12). The need for more data is clearly evident. For their collection certain well known facts can be utilized.

In 1908 Windaus (13) demonstrated that cholesterol in solution in alcohol will combine with digitonin to form an insoluble compound. He showed further that other sterols (phytosterol, stig-

[1] Unpublished data.

masterol, coprosterol, and β-cholestanol) would form similar insoluble compounds. This work has since been abundantly confirmed by various investigators and numerous applications in qualitative and quantitative analyses have been made of it. Lewkowitsch (14) includes brassicasterol among the sterols precipitable by digitonin. Gardner and his coworkers (15) have made use of the above reaction in their extensive work on cholesterol absorption and excretion, and Bloor and Knudson (16) have also used it in their excellent studies on the cholesterol changes in the blood.

The use of digitonin to remove cholesterol from the non-saponifiable fraction of cod liver oil would immediately suggest itself as a means of effecting its quantitative separation from extracts containing both vitamin A and the antirachitic factor and then determining the biological properties of the residual materials by means of rat feeding experiments.

## EXPERIMENTAL.

### *I. Observations on the Removal of the Antirachitic Factor as Revealed by Growth in the Rat.*

The biological tests of the materials in question were made on young growing rats taken from our stock colony. Our stock animals had been kept on rations which insured the production of young particularly suitable for these experiments—some on a ration not excessively provided with fat-soluble compounds and others receiving vitamin A additions. Rats from 21 to 23 days old and weighing from 50 to 55 gm. were used for these experiments. They were kept in our standard cages provided with removable galvanized iron pan bottoms provided with screens to prevent access to excreta. They were put upon a ration free from fat-soluble vitamins, consisting of alcohol-extracted and heated casein, 18; salt 40 (12), 4; agar, 2; yeast, 6; and dextrin, 70. Distilled water was provided in glass drinking fountains. At the desired time, solutions of preparations from cod liver oil were evaporated on the basal ration. This was done weekly and the rations were kept in brown bottles to minimize their deterioration. Each rat was weighed weekly and careful records were kept of visible changes in their condition.

The non-saponifiable fraction of cod liver oil was prepared as follows: 250 gm. of oil were boiled for 30 minutes with 500 cc. of a 20 per cent solution of KOH in ethyl alcohol. After dilution with 2.5 liters of water, the soaps were thoroughly shaken with 1 liter of ether. After ½ hour about 500 cc. of ether were separated off. Two successive extractions, each of 1 hour duration, were then made with two portions of 500 cc. of ether each. The extracts were combined, washed with 500 cc. of water, and distilled down to a volume of 50 to 100 cc. The remaining solvent was then dissipated in a dish before a fan. To remove all question as to completeness of saponification of this preparation, the residue was taken up in alcohol and boiled for ½ hour with 250 cc. of a 4 per cent solution of potassium hydroxide in alcohol. After cooling, an equal volume of water was added and extraction made with three 200 cc. portions of petroleum ether (60–90°C.). All operations were carried out in a room with curtains drawn to exclude sunlight which we repeatedly have found destroys vitamin A in concentrated preparations very rapidly.

In using digitonin for the preparation of extracts to be fed to animals, it was obviously necessary to take cognizance of the fact that digitonin, though not actively toxic, nevertheless produces very marked physiological effects upon the organism. Ransom (17) found that the minimum quantity of digitonin necessary under specified conditions to lake ox blood completely corresponded to a dilution of 1:136,000 to 1:139,000. Evaporation to dryness on the water bath did not affect this hemolytic power. Perfusion of a frog's heart *in situ* with a 0.001 per cent solution produced an increase in the beat followed by a rise in tone, diminution of diastole, and finally approximate inhibition of beat. Fortunately he presents evidence as follows: "mono molecular combinations of digitonin with phytosterol, coprosterol and beta cholestanol are non toxic for red cells and for frog's heart; any excess of digitonin beyond this proportion can be detected by the action of the solution upon red cells and upon the heart beat."

We have prepared fractions approximately freed from the cholesterol of cod liver oil as follows: an aliquot of our petroleum ether solution of the non-saponifiable fraction of cod liver oil prepared as previously described equivalent to 50 gm. of cod liver oil was evaporated just to dryness and then dried *in vacuo* over-

night. The residue weighing 0.4168 gm. was taken up in 10 cc. of hot alcohol, and then 55 cc. of a hot 1 per cent solution of digitonin in 90 per cent alcohol were added slowly and with constant stirring. After standing 4 hours in the ice box the solution was filtered. The filtrate on testing showed incomplete removal of cholesterol, so after concentrating it to 20 cc., 30 cc. more of the digitonin solution were added. It was then allowed to stand overnight in the ice box and filtered. Qualitative tests now showed the presence of an excess of digitonin. To remove this, we added 10 cc. of hot alcohol, containing 0.12 gm. of cholesterol prepared from sheep's brain and allowed the solution to stand in the ice box until the following morning. It was then filtered, the filtrate evaporated to dryness on the water bath and taken up in ether. The dried digitonin cholesteride removed in the first two percipitations weighed 0.8508 gm., hence there must have been 0.2074 gm. or 50.4 per cent of cholesterol in the non-saponifiable fraction.

These preparations, the non-saponifiable fraction of cod liver oil in one case, and the same after the removal of sterols with digitonin in the other, were fed to rats that had been on a ration free from vitamin A and the antirachitic factor for 2 weeks. They were incorporated in the ration in amounts equivalent to 5 per cent of cod liver oil. To insure that no active constituent had been added with the cholesterol used for removing excess of digitonin, it was also fed to some animals at a level of 1 per cent of the weight of the ration. Chart I shows the growth response obtained as a result of these additions. Both preparations were found equally active. The cholesterol was inactive.

To interpret these results a word or two of explanation may be in order which will briefly recapitulate our views on the relation of vitamins to growth. We believe that all vitamins are necessary for growth and that therefore there is no particular reason for speaking of any one of them as the "growth vitamin." We are convinced that when a rat does not grow for some weeks in spite of an absence of ophthalmia or respiratory trouble and in spite of the fact that it is provided with an abundance of vitamin B, good protein, salts, and calories, that failure of growth is then due to a deficiency of the antirachitic factor. We are supported in this belief by the fact that irradiation of the rat will promptly

XUM

reinitiate growth (12), and with this treatment the content of the tissues of the rat in antirachitic factor is increased. Additional amounts of vitamin A will not elicit such a reaction. These responses are therefore taken to indicate that the cod liver oil preparation was not deprived of its antirachitic potency by the removal of its cholesterol through the usual method of precipitation by digitonin.

This experiment was later repeated with some modifications. Non-saponifiable fractions prepared from four 250 gm. portions of cod liver oil in petroleum ether were combined and then divided

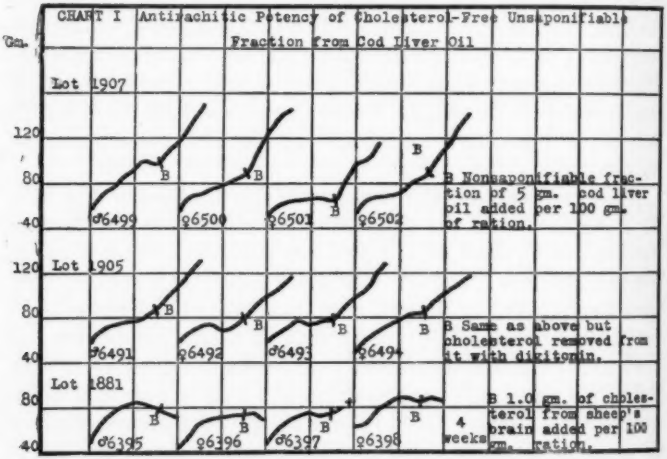


CHART I. Removal of the cholesterol from the non-saponifiable fraction of cod liver oil by precipitation with digitonin has no apparent effect on its antirachitic potency when fed at a level equivalent to 5 per cent of cod liver oil. Cholesterol prepared from sheep's brain and used to remove excess digitonin from the preparation fed had no power to stimulate growth.

into two equal parts *A* and *B*. *A* was evaporated to dryness; the residue heated 10 minutes on a water bath; then dried ½ hour in a vacuum desiccator; and weighed. The residue weighing 3.8839 gm. was taken up in alcohol and as much cholesterol as possible removed by crystallization. 1.49 gm. were obtained. The filtrates and washings were preserved and combined. Finally the alcohol was evaporated off and the residue taken up in 250

cc. of petroleum ether. 1 cc. of this solution was equivalent to 2 gm. of cod liver oil. This solution will hereafter be referred to as "solution *A*." Part *B* from which the cholesterol was not removed by crystallization in this manner will be referred to as "solution *B*."

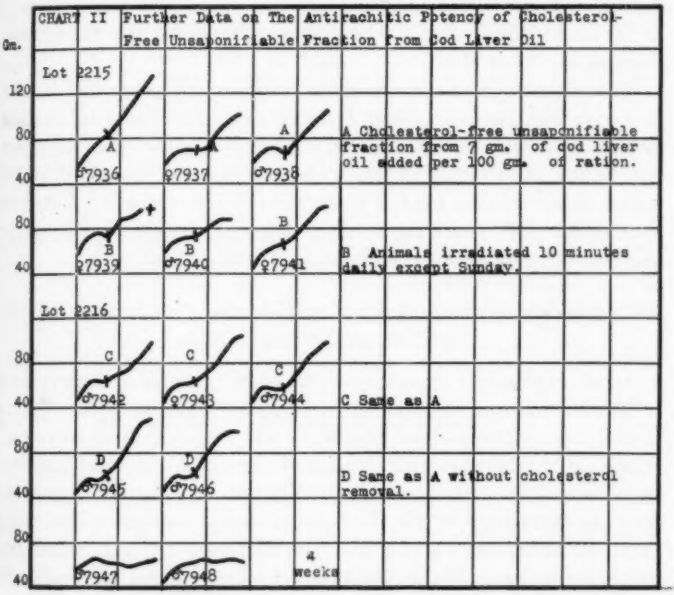


CHART II. Further evidence of the antirachitic potency of the non-saponifiable fraction of cod liver oil from which the cholesterol had been removed by digitonin is provided by the growth curves of Rats 7936, 7937, and 7938 of Lot 2215 and Rats 7942, 7943, and 7944 of Lot 2216. The growth response of these animals was on a parity with that of Rats 7945 and 7946 which received the same preparation except that the cholesterol had not been removed. Supplying the antirachitic factor by exposing one-half of Lot 2215 to ultra-violet light initiated a growth response, but this was limited by a deficiency of vitamin A. The control animals, Nos. 7947 and 7948, made no appreciable gain in weight after the end of the 2nd week.

Treatment of "solution *A*" was continued as follows: 50 cc. were evaporated to dryness over a water bath and the residue was taken up in 10 cc. of hot alcohol. 25 cc. of a hot 1 per cent

solution of digitonin in 90 per cent alcohol were then slowly added. After cooling for 4 hours in the ice box the solution was filtered and 5 cc. additional digitonin solution were added to test out the completeness of the cholesterol removal. On filtering in the morning the filtrate showed an excess of digitonin. This was removed by adding 0.1 gm. of inactive cholesterol—prepared from brain—in 10 cc. of hot alcohol. The following morning the precipitate formed was filtered off.

The filtrate of the above and "solution *B*" were fed in the amounts designated in Chart II to rats that had been on a ration free from fat-soluble vitamins for 3 weeks. As shown in the chart the antirachitic factor which is the principle in the cod liver oil which is responsible for the promotion of growth, when added to our ration free from fat-soluble vitamins, is not destroyed or removed by the removal of its sterols in this manner.

*II. Observations on the Removal of Vitamin A as Indicated by the Ophthalmic Reaction in Rats.*

In the foregoing experiments on growth, careful observations were also taken on the occurrence and cure of ophthalmia. Tables I and II summarize these findings. The prevention, or more particularly the cure, of this deficiency disease is the most satisfactory criterion of the presence of vitamin A. As an alternative one can also use growth as the index when the animals are provided with an abundance of the antirachitic factor by irradiation and with a sufficiency of vitamin B, good protein, salts, and sufficient calories in the ration. This technique, however, was not used in these experiments.

As Table I shows in summary form, six animals having sore eyes before the ration was changed were rapidly cured when the cod liver oil fractions were introduced. Two of these cases (Rats 6500 and 6494) were not severely afflicted, but the rapid cures of the other infected animals speak for the presence of an abundance of vitamin A in the preparation. The eyes of Rat 6501 were questionable when the ration was changed, but they appeared quite normal the following week. No evidence of any decrease in potency in the fraction treated with digitonin to remove sterols was obtained. The prophylactic experiments in Lots 2215 and

TABLE I.

*Cure of Ophthalmias in Rats by the Administration of the Unsaponifiable Constituents of Cod Liver Oil. (See Chart I for Growth).*

| Lot No. | Condition of animals when changed. | | Condition of animals after change. | | Dietary additions with change of ration. |
|---|---|---|---|---|---|
| | Ophthalmia developed in Rat No. | Ophthalmia not developed in Rat No. | Ophthalmia cured in Rat No. | Ophthalmia not cured in Rat No. | |
| 1907 | 6499 | 6501(?). | 6499 | | Unsaponifiable fraction from 5 gm. of cod liver oil. |
| | 6500 | | 6500 | | |
| | 6502 | | 6502 | | |
| 1905 | 6491 | 6492 | 6491 | | Unsaponifiable fraction from 5 gm. of cod liver oil. Sterols removed by digitonin. |
| | 6493 | | 6493 | | |
| | 6494 | | 6494 | | |
| 1881 | 6395 | | | 6395 | 1.0 gm. of cholesterol from brain. |
| | 6396* | | | 6396 | |
| | 6397 | | | 6397 | |
| | 6398* | | | 6398 | |

*Developed ophthalmia shortly after change of ration.

TABLE II.

*Prevention of Ophthalmias in Rats by the Administration of the Unsaponifiable Constituents of Cod Liver Oil. (See Chart II for Growth).*

| Lot No. | Ophthalmias incident in Rat No. | Ophthalmias not incident in Rat No. | Dietary adjuvant or animal treatment. |
|---|---|---|---|
| 2215 | 7939 | | Rats irradiated. |
| | 7940 | | |
| | 7941 | | |
| | | 7936 | The non-saponifiable fraction of 7 gm. of cod liver oil treated with digitonin to remove sterols. |
| | | 7937 | |
| | | 7938 | |
| 2216 | 7947 | | None. |
| | 7948 | | |
| | | 7942 | The non-saponifiable fraction of 7 gm. of cod liver oil treated with digitonin to remove sterols. |
| | | 7943 | |
| | | 7944 | |
| | | 7945 | The non-saponifiable fraction of 7 gm. of cod liver oil. |
| | | 7946 | |

2216 (Table II) provide additional evidence in confirmation of this conclusion.

To obtain evidence on the stability of vitamin A in petroleum ether solution the vitamin A activity of "solution *A*" was again tested 1½ years later. The sterols were removed with digitonin as before and the same sample of cholesterol was used to remove excess of digitonin. Two rats were cured of severely infected eyes in a period of 10 days by introducing an equivalent of 5 per cent of cod liver oil into our ration.

*III. Observations on the Removal of the Antirachitic Factor from Cod Liver Oil as Revealed by Cure of Rickets in Rats.*

We have supplemented our experiments using growth for estimating potency in antirachitic factor by experiments on rickets itself. Rickets was produced in our rats by feeding them—after reaching a weight of approximately 50 gm.—for about 4 weeks on our laboratory ration No. 2966. This consists of yellow corn 71, wheat gluten 20, gelatin 5, calcium carbonate 3, and sodium chloride 1. We have also used a similar ration, No. 2965, in which we replaced the gelatin of No. 2966 with an equal amount of yellow corn. These rations have proven more satisfactory than the McCollum (18) or the Sherman and Pappenheimer (19) rations, which seem to have been used so successfully by their originators. The desired addition of extracts was made to these rations for a period of 10 days during which time records were kept on the consumption of ration as well as body weights. At the end of this time the ulnæ and radii were removed; their distal ends split with a scalpel and stained with silver nitrate (20). The extent of calcification of the rachitic lesions as induced by the supplementation of the ration with the extracts was readily determined with the naked eye.

The results of these experiments are summarized in Table III. Here "positive" in the column headed "Calcification" means complete calcification of the rachitic lesion, and "negative" means absence of calcification. Four rats in Lots 2999 and 3000 received a preparation made from "solution *A*" by removing the sterols with digitonin and four rats an equivalent amount of "solution *B*"—the non-saponifiable fraction corresponding to "solution *A*" but

still containing its cholesterol. The results were very uniform and unequivocal. We attribute a great deal of the uniformity of results to the character of the basal ration. Severe rickets was always produced at an early age and to a uniform degree. The results were not disturbed by failure of consumption or by complication of vitamin A deficiency which was prevented by the large amount of yellow corn in the ration.

TABLE III.

*Calcification of Rachitic Lesions by Cod Liver Oil Preparations.*

| Lot No. | No. of rats. | Additions to basal ration. | Calcification. |
|---|---|---|---|
| 2999 and 3000 | 4 | 5 per cent cod liver oil equivalent, as unsaponifiable fraction. | Positive. |
| | 4 | 5 per cent cod liver oil equivalent, as unsaponifiable fraction but freed from cholesterol. | " |
| | 4 | No additions. | Negative. |
| 3066 and 3070 | 2 | 2 per cent cod liver oil equivalent, as unsaponifiable fraction freed from cholesterol. | Positive. |
| | 2 | 5 per cent cod liver oil equivalent, as unsaponifiable fraction freed from cholesterol. | " |
| | 2 | 0.1 per cent "fresh" cholesterol. | Negative. |
| | 1 | 0.2 " " "old" cholesterol. | " |
| | 1 | 0.2 " " " " irradiated. | Positive. |
| | 2 | No additions. | Negative. |

It was a question in our minds if we could grant that digitonin removed the cholesterol and other sterols sufficiently quantitatively from our preparation to eliminate them as possible factors in the reaction produced in our animals. That cholesterol digitonide is appreciably soluble in alcohol was granted even by Windaus. He expressed the following relations (13): 100 cc. of 95 per cent ethyl alcohol dissolve 0.014 gm. at 18°C. and 0.16 gm. at 78°C. 100 cc. of boiling 50 per cent ethyl alcohol dissolve 0.03 gm. It is slightly soluble in pyridine and insoluble in cold water, acetone, ether, acetic acid, and benzene.

Wacker and Hueck (21) report that 100 cc. of 94.8 per cent alcohol dissolve 0.024 gm. of the digitonide; 92.2 per cent alcohol, 0.024 gm.; ether shaken with water and alcohol, 0.008 gm.; and petroleum ether, 0.0023 gm. The amount of cholesterol dissolved in these combinations would be approximately equal to one-fourth of the above figures. Bloor and Knudson (16) made use of the insolubility of the digitonide in petroleum ether by evaporating the extract containing the excess of digitonin to dryness and then extracting with this solvent. We have found that the cholesterol digitonide is somewhat soluble in petroleum ether but far less so than in alcohol, approximately in the ratio of 1:20. As a refinement in our procedure for the preparation of an extract free from cholesterol, we have seen fit to use this solvent. After the addition of an excess of digitonin to the unsaponifiable fraction equivalent to 15 gm. of cod liver oil dissolved in alcohol, we evaporated it to dryness and then extracted it three times with 10 cc. quantities of petroleum ether. These extracts were united, freed from petroleum ether over a water bath, and then taken up in alcohol to be evaporated on the ration.

When fed in amounts equivalent to 2 and 5 per cent of cod liver oil, the above preparation caused complete calcification of the rachitic metaphyses in the short period of 10 days. Apparently then, digitonin cannot remove the antirachitic factor from cod liver oil, but this should not be interpreted as proof that the preparation was free from sterols. As was stated before, too little is known of the nature of the compounds found in this fraction to warrant such conclusions. It is interesting to note that after the digitonin precipitation the extract still contained compounds which gave a color reaction with the Liebermann-Burchard test. This, however, differed in character from that given by cholesterol.

The preparation was equally efficacious in curing ophthalmia. Three animals which had developed marked ophthalmias after living on a ration free from vitamin A and the antirachitic factor were completely cured in 8 to 11 days when an equivalent of 5 per cent of cod liver oil was added to the diet.

The activity of the cholesterol removed from cod liver oil in preparing our "solution *A*" and of cholesterol removed from "solution *B*" was also tested. The cholesterol obtained in preparing

"solution *A*" had been left standing in a covered crystallizing dish for over 18 months. After recrystallizing from alcohol one portion of it was fed directly and another portion after irradiation with ultra-violet light in ether solution. The irradiated material proved to be very potent while the untreated was inactive even when fed at a 0.2 per cent level in the ration. This inactivity was not due to aging of the dry preparation for a freshly prepared sample of cholesterol from "solution *B*" was also found inactive.

## CONCLUSIONS.

The compounds responsible for the antiophthalmic and antirachitic properties of cod liver oil are non-precipitable by digitonin.

A petroleum ether solution of the unsaponifiable constituents of cod liver oil standing for a year and a half in the laboratory did not lose its antiophthalmic or antirachitic potency.

Cholesterol isolated from cod liver oil was antirachitically inactive, but exposure to ultra-violet light made it active.

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# THE PRODUCTION OF ACIDOSIS BY ANOXEMIA.

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The conception that limitation of oxygen to the animal organism results in an acidosis is not new and has generally been accepted by physiologists for many years. There was, however, no experimental evidence that warranted the assumption that a change in the reaction of the blood or tissues is produced. In fact, the theory of acidosis in oxygen deficiency had practically its sole origin in the observations of Araki (1), Zillessen (2), and Ryffel (3) of the appearance of lactic acid in the blood and urine. That this was insufficient evidence has been emphasized by recent work, Macleod and Knapp (4), and Anrep and Cannan (5), showing that the presence of lactic acid in the urine or blood does not necessarily indicate an acidosis but may be the result of an alkalosis. Commenting upon the discovery of the appearance of lactic acid, Haggard and Henderson (6) stated, "Without this foundation of correct observation, but incorrect inference, the theory of acidosis in a wide variety of conditions would probably never have attained the hold which it now has on current thought."

The first measurements of blood reaction during oxygen want were made by Haggard and Henderson (7), using the indirect method devised by these workers of equilibration at various $CO_2$ tensions and calculating the pH from the free $CO_2$:fixed $CO_2$ ratio. These investigators found, not an acidosis, but an alkalosis. They attributed the increase in blood pH to the blowing off of $CO_2$ due to the anoxemic hyperpnea and so disturbing the $\frac{H_2CO_3}{BHCO_2}$ ratio. From these results they were led to the conclusion quoted from their paper entitled "The fallacy of asphyxial acidosis."

In a previous preliminary note, Koehler, Brunquist, and Loevenhart (8) showed that the observations of Haggard and Henderson were correct as far as they went, but that they failed to extend their experiments during anoxemia long enough to reveal the fact that oxygen deficiency results later in true acidosis. We agreed with Haggard and Henderson that the alkalosis noted by them is due to the overventilation and loss of $CO_2$; we pointed out, however, that the alkalosis is but temporary and this is soon offset and later overshadowed by a rapidly developing acidosis.

This paper deals with the method of experimentation and presents the data that warranted the above conclusions.

Rabbits and pigs were used for experimental animals. On account of their small size and small blood volumes rabbits were discarded and pigs were used in most of the experiments. Arterial blood can be drawn from the ear of a rabbit, but it is a slow and uncertain procedure, especially when the circulation is embarrassed by oxygen deficiency, and ventricular punctures had to be resorted to at times at the end of the experiments. It is very easy to obtain blood from the pig by cutting off a small section of the tail with a sharp chisel. Arterial blood can be collected directly under oil with a minimum of inconvenience to the animal. In a few pigs the circulation was so enfeebled at the end of the experiment that left ventricular punctures had to be resorted to. At that stage of anoxemia, the pigs were comatose and did not feel the puncture.

The decreased oxygen tension of the inspired air was obtained by placing the animal in a metal respiratory chamber and then reducing the oxygen content of the air and at the same time maintaining atmospheric pressure in the chamber by adding nitrogen. The $CO_2$ and moisture were eliminated by circulating the air through a soda-lime and sulfuric acid system. The oxygen in the atmosphere of the chamber was reduced (*a*) by respiration, (*b*) by means of burning a jet of hydrogen, (*c*) by dilution with nitrogen, or (*d*) by a combination of these methods. The chamber and the details of methods of reducing the oxygen content are discussed in detail in earlier publications from this laboratory (9). The pH of the blood was determined by the electrometric method, as already described by Koehler (10). The total $CO_2$ was determined directly on whole blood, drawn under oil, by

means of the Van Slyke constant pressure apparatus. The $CO_2$ tension of the whole blood was calculated by means of the Hasselbalch formula. A discussion of the factors used, their derivation, and the error of the method of calculation appear in a previous paper by Koehler (11).

Animals refuse to eat in an atmosphere of low oxygen content so that all experiments at low oxygen percentages were necessarily made on fasting animals and, therefore, in order to have a complete control it was necessary to have a previous period with each animal while breathing normal air and fasting. This was done in each experiment of sufficient duration for the fasting to be a factor in disturbing the acid-base equilibrium. In the shorter experiments the animals did not fast.

## RESULTS.

*Short Experiments.*—Table I shows the effects of comparatively short periods of oxygen deficiency. Pig 1 shows distinctly that if the oxygen percentage of the inspired air is rapidly reduced to the low value of 2.6 per cent in 7 to 8 hours, an extreme degree of acidosis is produced. Pig 2 shows this even more strikingly, the low pH value of 6.71 having been obtained. Another interesting feature is brought out in these two pigs, characteristic of all studies of the effects of low oxygen; namely, that of individual differences. Although Pig 1 was subjected to a lower oxygen percentage and for a period longer by several hours, it had a less severe acidosis than Pig 2. Pigs 3 and 4, exposed for approximately the same period of time to low oxygen tension but to less drastic levels, showed a distinct blood alkalosis. It is apparent that if the animals in the alkalotic state had been exposed to further decreasing amounts of oxygen, a marked acidosis would rapidly have developed. Experiments, which will be described later, further emphasize this point. It is of interest to note that Pig 5 shows a pH value at the end of the run that is within the normal range. Evidently the blood sample was obtained just at the time when the initial alkalosis was compensated by the developing acidosis.

The short period experiments gave a good opportunity to observe the effect of low oxygen tensions on the respiratory rate. There was a marked stimulation in all cases, the maximum rate varying from 130 to 200. If the oxygen deficiency were of sufficient duration and severity the respiration became depressed again, slowly at first, but finally very rapidly. In certain animals only a very short period of time supervenes between the break in the respiratory center and death. In none of the animals that showed an alkalosis was the respiration depressed at any time. Tables II and III show the initial stimulation and final depression of respiration together with the oxygen percentage and pH changes.

*Long Experiments.*—Table IV shows the results of long periods of oxygen deficiency on the rabbit. In one experiment six rabbits were exposed for 26 hours to 15.0 to 4.2 per cent of oxygen, and in the other three rabbits were exposed for 120 hours to 7.7 to 5.1 per cent. The rabbits exposed for only 26 hours showed but little change in the blood pH but showed a marked decrease in the total $CO_2$ and in the $CO_2$ tension, while the rabbits exposed for 120 hours in addition showed a definite change in the pH of the blood

TABLE I.

*Short Periods—Pigs.*

| Animal No. and sex. | Weight. | Conditions. | Range of $O_2$ content. | Duration of period. | | Respiratory rate. | | Blood. | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Normal. | Maximum. | Total $CO_2$. | Tension $CO_2$. | pH |
| | *kg.* | | *per cent* | *hrs.* | *min.* | | | *vol. per cent* | *mm. Hg* | |
| 1 | | Normal $O_2$. | | | | 24 | | 58.8 | 48.1 | 7.44 |
| Male. | 7.6 | Reduced $O_2$. | 20.9–2.6 | 7 | 48 | | 180 | 17.6 | 42.5 | 6.91* |
| 2 | | Normal $O_2$. | | | | 22 | | 51.9 | 48.6 | 7.38 |
| Female. | 7.9 | Reduced $O_2$. | 20.9–3.2 | 4 | 48 | | 165 | 9.8 | 32.0 | 6.71* |
| 3 | | Normal $O_2$. | | | | 28 | | 52.5 | 43.8 | 7.43 |
| Male. | 9.1 | Reduced $O_2$. | 20.9–7.6 | 5 | | | 150 | 45.0 | 29.1 | 7.54* |
| 4 | | Normal $O_2$. | | | | 30 | | 49.1 | 45.2 | 7.39 |
| Male. | 11.1 | Reduced $O_2$. | 20.9–5.7 | 6 | | | 130 | 36.1 | 24.6 | 7.52* |
| 5 | | Normal $O_2$. | | | | 26 | | 53.2 | 47.2 | 7.40 |
| Male. | 7.8 | Reduced $O_2$. | 20.9–4.8 | 6 | | | 200 | 34.8 | 32.6 | 7.38 |

* Blood from left ventricle.

toward the acid side. One animal, Rabbit 3, apparently healthy before the experiment, died toward the end of the run. Table V gives the results of long experiments with pigs. In these experiments the oxygen percentage was not so low, averaging about 7 per cent, as in the short runs. All animals except Pig 1 developed a definite acidosis; in one case the pH dropped as low as 6.90. Pig 1 was exposed to an average of about 9 per cent of oxygen for 46 hours and showed at the end of the run a mild blood alkalosis.

TABLE II.

*Pig 1 of Table I.*

| Time. | | $O_2$ | Respiratory rate. | Blood. | | |
|---|---|---|---|---|---|---|
| | | | | Total $CO_2$. | Tension $CO_2$. | pH |
| *hrs.* | *min.* | *per cent* | | *vol. per cent* | *mm. Hg* | |
| 0 | | 20.2 | 24 | 58.8 | 48.1 | 7.44 |
| | 35 | 15.5 | | | | |
| 1 | 16 | 13.3 | 27 | | | |
| 1 | 50 | 11.0 | 24 | | | |
| 2 | 50 | 8.9 | | | | |
| 3 | 57 | 7.0 | 84 | | | |
| 4 | 40 | 6.9 | 90 | | | |
| 5 | 9 | 6.5 | 94 | | | |
| 5 | 43 | 6.4 | 90 | | | |
| 6 | 10 | 6.1 | 84 | | | |
| 6 | 40 | 5.6 | 74 | | | |
| 6 | 55 | | 122 | | | |
| 7 | 5 | 4.4 | 160 | | | |
| 7 | 15 | 3.6 | 180 | | | |
| 7 | 26 | 2.8 | 146 | | | |
| 7 | 36 | 2.6 | 98 | | | |
| 7 | 39 | 2.6 | 20 | | | |
| 7 | 44 | | 4 | 17.6 | 42.5 | 6.91 |

TABLE III.

*Pig 2 of Table I.*

| Time. | | $O_2$ | Respiratory rate. | Blood. | | |
|---|---|---|---|---|---|---|
| | | | | Total $CO_2$. | Tension $CO_2$. | pH |
| *hrs.* | *min.* | *per cent* | | *vol. per cent* | *mm. Hg* | |
| 0 | | 20.1 | 22 | 51.9 | 48.6 | 7.38 |
| | 12 | 11.8 | | | | |
| | 34 | 8.3 | 28 | | | |
| 1 | 5 | 7.2 | 59 | | | |
| 2 | 17 | 6.7 | 65 | | | |
| 3 | 20 | 5.9 | 60 | | | |
| 3 | 57 | 4.8 | 106 | | | |
| 4 | 15 | | 114 | | | |
| 4 | 19 | 4.2 | | | | |
| 4 | 23 | | 165 | | | |
| 4 | 32 | 3.4 | 120 | | | |
| 4 | 45 | 3.2 | 26 | | | |
| 4 | 48 | | 6 | 9.8 | 32.0 | 6.71 |

TABLE IV.
*Rabbits.*

| Animal No. | Conditions. | Range of $O_2$ content. | Duration of period. | Blood. | | |
|---|---|---|---|---|---|---|
| | | | | Total $CO_2$. | Tension $CO_2$. | pH |
| | | *per cent* | *hrs.* | *vol. per cent* | *mm. Hg* | |
| 1 | Normal $O_2$. | | | 55.4 | 40.6 | 7.49 |
| | Reduced $O_2$. | 15.0–4.2 | 26 | 32.6 | 32.7 | 7.35 |
| 2 | Normal $O_2$. | | | 56.3 | 49.8 | 7.40 |
| | Reduced $O_2$. | 15.0–4.2 | 26 | 29.2 | 29.8 | 7.34 |
| 3 | Normal $O_2$. | | | 45.5 | 40.3 | 7.40 |
| | Reduced $O_2$. | 15.0–4.2 | 26* | | | |
| 4 | Normal $O_2$. | | | 48.7 | 44.8 | 7.39 |
| | Reduced $O_2$. | 15.0–4.2 | 26 | 33.2 | 31.1 | 7.38 |
| 5 | Normal $O_2$. | | | 50.4 | 42.1 | 7.43 |
| | Reduced $O_2$. | 15.0–4.2 | 26 | 28.6 | 31.2 | 7.31 |
| 6 | Normal $O_2$. | | | 51.5 | 44.8 | 7.41 |
| | Reduced $O_2$. | 15.0–4.2 | 26 | 35.4 | | |
| 7 | Normal $O_2$ and diet. | | 72 | 52.3 | 39.3 | 7.48 |
| | " " " fasting. | | 96 | 44.5 | 37.9 | 7.42 |
| | Reduced " " " | 7.7–5.1 | 120 | 15.6 | 20.2 | 7.23 |
| 8 | Normal $O_2$ and diet. | | 72 | 59.9 | 43.8 | 7.49 |
| | " " " fasting. | | 96 | 38.3 | 39.2 | 7.34 |
| | Reduced " " " | 7.7–5.1 | 120 | 25.2 | 28.3 | 7.30 |
| 9 | Normal $O_2$ and diet. | | 72 | 42.7 | 34.4 | 7.45 |
| | " " " fasting. | | 96 | 32.6 | 31.1 | 7.37 |
| | Reduced " " " | 7.7–5.1 | 120 | 19.1 | 22.1 | 7 28 |

*Died.

TABLE V.
*Long Runs—Pigs.*

TABLE V.
*Long Runs—Pigs.*

| Animal No. and sex. | Weight. | Conditions. | Range of $O_2$ content. | Duration of period. | Blood. | | |
|---|---|---|---|---|---|---|---|
| | | | | | Total $CO_2$. | Tension $CO_2$. | pH |
| | *kg.* | | *per cent* | *hrs.* | *vol. per cent* | *mm. Hg* | |
| 1 | | Normal $O_2$ and diet. | | 96 | 55.8 | 49.4 | 7.40 |
| Male. | 7.6 | " " " fasting. | | 48 | 55.7 | 47.4 | 7.42 |
| | | Reduced " " " | 10.0–7.9 | 46 | 37.0 | 27.7 | 7.48 |
| 2 | | Normal $O_2$ and diet. | | 96 | 53.3 | 49.4 | 7.39 |
| Female. | 16.2 | " " " fasting. | | 96 | 47.3 | 41.2 | 7.41 |
| | | Reduced " " " | 10.2–4.0 | 96 | 24.5 | 30.4 | 7.26 |
| 3 | | Normal $O_2$ and diet. | | 72 | 56.2 | 49.8 | 7.40 |
| Female. | 8.0 | " " " fasting. | | 72 | 53.8 | 49.5 | 7.39 |
| | | Reduced " " " | 12.6–5.6 | 110 | 38.1 | 38.3 | 7.35 |
| 2 | | Normal $O_2$ and diet. | | 48 | 54.3 | 44.4 | 7.44 |
| Female (second experiment). | 16.0 | " " " fasting. | | 96 | | | |
| | | Reduced " " " | 8.3–6.9 | 70 | 11.8 | 28.6 | 6.90 |
| 3 | | Normal $O_2$ and diet. | | 72 | 45.6 | 38.2 | 7.43 |
| Female (second experiment). | 9.0 | " " " fasting. | | 72 | 50.2 | 46.4 | 7.39 |
| | | Reduced " " " | 8.5–6.2 | 96 | 24.4 | 34.1 | 7.20 |
| 4 | | Normal $O_2$ and diet. | | 72 | 48.4 | 41.3 | 7.42 |
| Male. | 9.2 | " " " fasting. | | 48 | | | |
| | | Reduced " " " | 8.5–7.2 | 42 | 44.0 | 44.9 | 7.34 |
| 5 | | Normal $O_2$ and diet. | | 96 | 42.8 | 32.2 | 7.48 |
| Male. | 12.8 | " " " fasting. | | 96 | 17.4 | 19.6 | 7.30 |
| | | Reduced " " " | 7.7–7.5 | 66 | (Died.) | | |

DISCUSSION.

It is now fairly well understood among workers in this field that the term acidosis is limited to those conditions where the blood pH is lower than the normal limits of variation. Similarly, the term alkalosis is used to designate an increase in the blood pH. Our data clearly show that the change in the blood reaction is diphasic if anoxemia is carried sufficiently far. The initial alkalosis agrees with the findings of Haggard and Henderson. It is directly the result of the hyperpnea caused by oxygen deficiency and in that manner only indirectly the result of diminished oxidation. The ease and rapidity with which a large amount of $CO_2$ can be eliminated by moderate overbreathing with the marked alkalosis resulting, is now a well known fact. We believe that in anoxemia the true acidotic nature of diminished oxidation is merely masked at the beginning by the initial alkalosis and that were it not for the production of fixed acids, the shift of the pH during the initial period would be even more marked toward the alkaline side. In a future communication one of us (Koehler) will show that for the same increase in volume respiration in man, voluntary hyperpnea with ordinary air results in a higher pH of the blood than a similar hyperpnea resulting from oxygen deficiency. We believe that acid products are formed within the cell as soon as the process of oxidation is handicapped by oxygen deficiency, but this increased production of fixed acids cannot at first compensate for the loss of $CO_2$. The pH of the blood observed **at** any phase represents the algebraic sum of the two factors; *viz.*, increase in non-volatile acids and loss of $CO_2$. If the oxygen deficiency is of sufficient grade and endures for a sufficient period of time, the acid products formed in the tissues pass into the blood and overcome the blood alkalinity with the resulting marked acidosis described. The acidosis so induced is, as far as we are aware, the most severe ever observed and of a magnitude heretofore considered altogether incompatible with life. The statement has often been made that hemoglobin cannot function as a transporter of oxygen on the acid side of its isoelectric point, pH 6.8. Although we have obtained whole blood reactions as low as pH 6.5 with recovery, it is rather precarious to assume that the reaction of the hemoglobin within

the blood cells was identical with the figure found for the whole blood because we do not know how long this low pH persisted nor whether the time was sufficient for complete equilibration.

Our figures in regard to the total $CO_2$ during anoxemia are similar to those of Haggard and Henderson except that they did not obtain the extreme low values (as low as 10 volumes per cent in the most extreme cases) that we have found in marked oxygen deficiency. Such low values could not be the result of overventilation, no matter how severe, and in themselves prove the presence of non-volatile acids in large amounts.

The change in the $CO_2$ tension of the blood during progressive anoxemia is also diphasic, in that it is at first markedly reduced and then rises toward the normal level. The rapid initial drop in $CO_2$ tension is the direct result of the hyperpnea and the alkalosis. As the acid products gradually enter the blood stream and neutralize the blood alkali, the dissociation of the $BHCO_3$-$H_2CO_3$ system is altered so as to increase the $CO_2$ tension. In the normal animal the feeding of acid produces only a slight rise in the $CO_2$ tension. In oxygen deficiency the acidosis occurs only after the period of hyperpnea when the total $CO_2$ is much reduced. In this case, therefore, the $CO_2$ tension rises above its previous low level during the alkalosis, but does not as a rule reach the normal level and in many cases remains far below normal.

*Respiration.*—Unfortunately it was only possible to observe the respiratory rate as we could not determine respiratory volume of the animals in the respiratory chamber. However, rate is a good criterion of ventilation when the magnitude of change ranges from 400 to 800 per cent, as occurred in our animals. The respiratory rate in such experiments is the best criterion of the condition of the animal. All animals show first the increased respiratory rate up to a maximum which may be maintained for minutes, hours, or days, depending on the degree of oxygen deficiency. Finally, if the oxygen want is sufficiently severe, the respiratory center breaks and the rate decreases very rapidly. It is then necessary to observe the rate continuously as the respiration may cease entirely within a few minutes, and unless the animal is removed before this occurs and artificial respiration in the air instituted, the animal will not survive. Death is due

to paralysis of the respiratory center and the heart continues to beat for several minutes.

In their article previously referred to, Haggard and Henderson present arguments why *a priori* doubt should have been aroused as to the correctness of the acidotic theory in asphyxia. The answer to all these arguments is that the acidosis actually occurs. These authors apparently assume that if there were an asphyxial acidosis, it would have to be caused by lactic acid, and then present their reasons why it is impossible that lactic acid should be the cause of the acidosis and hyperpnea. In regard to the nature of the acids arising in asphyxial acidosis, it is but natural that lactic acid should have played an important part since it was first observed in conditions of decreased oxidation and since it is so readily formed by the tissues. We do not presume to state what is the nature of the acidity. Although we have found lactic acid in the urine and blood in nearly all of our experiments, the amounts have always been relatively small. This is also true of the acetone bodies. The older methods for the determination of lactic acid are exceedingly faulty. It is quite probable that a large portion of the acidity is due to a disturbance in the phosphate system as is believed by Stehle and Bourne (12) to be the case in the acidosis of ether anesthesia. Other unknown acids may also play a rôle in asphyxial acidosis.

## CONCLUSIONS.

Anoxemia is fundamentally of an acidotic nature as far as disturbances in the acid-base balance are concerned. Acid production undoubtedly begins within the cell as soon as it suffers from oxygen deficiency. This condition is masked by the development of an alkalosis of the blood due to the blowing off of $CO_2$ during the initial period of overventilation.

The acidosis of anoxemia can be more marked (pH 6.7) than has been observed in any other condition. The available blood alkali may be depressed to extremely low limits (total $CO_2$ 9.8 volumes per cent) while the $CO_2$ tension, although greatly lowered at first, gradually rises toward the normal as the acidosis progresses.

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# THE RELATION OF HISTIDINE AND ARGININE TO CREATINE AND PURINE METABOLISM.*

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In a recent paper from this laboratory (Rose and Cox, 1924) it has been shown that histidine is an indispensable component of the diet, and that contrary to the observations of Ackroyd and Hopkins (1916), arginine and histidine are not mutually interchangeable for purposes of growth. In animals deprived of histidine the addition of arginine to the ration is without influence upon the rate of loss in body weight. On the other hand, the inclusion of histidine in the deficient diet occasions an immediate resumption of growth at a normal rate.

In their original communication, Ackroyd and Hopkins concluded that arginine and histidine are interchangeable also as precursors of allantoin. They found that when rats were fed diets devoid of arginine and histidine, but adequate in every other respect to meet the needs of metabolism, the elimination of allantoin decreased 40 to 50 per cent. The addition of either arginine or histidine to the deficient ration was said to result in a much smaller decrease in allantoin excretion than when both of the amino acids were wanting. Tryptophane deficiency was found to produce very little effect upon the allantoin output, although in some cases the losses in body weight were greater than those induced by arginine-histidine deficiency.

* This communication was presented in abstract before the American Society of Biological Chemists at Washington, D. C., December, 1924. See Rose, W. C., and Cook, K. G., *J. Biol. Chem.*, 1925, lxiii, p. xvii.

† The experimental data in this paper are taken from a thesis submitted by K. G. Cook in partial fulfillment of the requirements for the degree of Master of Science in Physiological Chemistry in the Graduate School of the University of Illinois.



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Inasmuch as the interchangeability of the two diamino acids as purine precursors seemed scarcely compatible with the results of our growth experiments, it appeared necessary to test the influence of the amino acids in question upon allantoin excretion. We have extended the observations to include uric acid and total creatinine.

## EXPERIMENTAL.

Growing rats served as the experimental animals. Each rat was confined in a separate cage, and its food consumption was carefully recorded. The diets employed are indicated in Table I, and are identical with those of like numbers described in the paper of Rose and Cox (1924). The inorganic portion of the food was supplied in the form of Osborne and Mendel's salt mixture (1919). The nitrogenous materials were furnished respectively in the form of purified casein, completely hydrolyzed casein, and hydrolyzed casein from which arginine and histidine had been precipitated by the Kossel-Kutscher procedure.[1] Purified amino acids were added where necessary as indicated in Table I. 25 mg. of Harris yeast vitamin were fed separately to each animal daily. In the experiments involving the feeding of arginine or histidine, the amino acid in question was added to Diet 11. Under such circumstances, 1.40 gm. of arginine nitrate, or 1.19 gm. of histidine monochloride, were employed per 100 gm. of food. Each of these quantities is equivalent to the sum of the arginine and histidine present in casein. The details followed in the preparation of the hydrolyzed casein, and of the hydrolyzed casein from which arginine and histidine were precipitated, have already been described elsewhere (Rose and Cox, 1924).

In the quantitative collection of the urines a procedure was used similar to that recommended by Ackroyd and Hopkins (1916). For this purpose the cages were supported in large funnels over the collecting beakers. In the latter were placed in-

[1] These materials were prepared by Mr. G. J. Cox of this laboratory. The experimental animals were also under his immediate care, and were being used in connection with other investigations, the results of which will be published later.

verted 100 cc. round bottom flasks to the necks of which small pedestals had been sealed. The urines flowed down the outer surfaces of the flasks into the beakers, while most of the solid materials were deflected in striking the inverted flasks and did not fall into the urine vessels. The funnels were washed down twice daily with dilute boric acid solution, and the urines and washings were preserved under toluene in an ice chest. Unless distinctly acid, they were rendered so by the addition of a few drops of dilute acetic acid. At the expiration of 8 day intervals,

TABLE I.

*Composition of the Diets.*

| Composition. | Diet No. | | |
|---|---|---|---|
| | 7 | 12 | 11 |
| Casein | 14.70 | | |
| Completely hydrolyzed casein | | 14.05 | |
| "Deficient digest" (hydrolyzed casein which had been precipitated with $Ag_2SO_4$ and $Ba(OH)_2$) | | | 14.05 |
| Cystine | 0.30 | 0.30 | 0.30 |
| Tyrosine | | 0.45 | 0.45 |
| Tryptophane | | 0.20 | 0.20 |
| Dextrin | 40.00 | 40.00 | 40.00 |
| Sucrose | 15.00 | 15.00 | 15.00 |
| Lard | 19.00 | 19.00 | 19.00 |
| Cod liver oil | 5.00 | 5.00 | 5.00 |
| Salt mixture | 4.00 | 4.00 | 4.00 |
| Agar | 2.00 | 2.00 | 2.00 |
| Total | 100.00 | 100.00 | 100.00 |

the urines of each animal were combined, diluted to a uniform volume of 600 cc., and immediately subjected to analysis. Total nitrogen was determined by the Kjeldahl-Gunning method, total creatinine by the procedure of Folin (1914), uric acid by the method of Benedict and Franke (1922), and allantoin according to the procedure of Wiechowski as modified by Handovsky (1914). In determining total creatinine and uric acid, standards were prepared of such concentrations as to approximate closely the strengths of the unknowns. Where necessary, preliminary analyses of the urines were made, and the standards diluted

accordingly. In most cases, 10 cc. samples of the urines were found satisfactory. After developing the colors, the solutions were diluted to volumes of 50 or 100 cc. as required. For the allantoin determinations, 400 cc. portions of the diluted urines were taken for the preliminary precipitations, and aliquots of

TABLE II.

*Nitrogen Metabolism on Diets of Whole Casein.*

| Period. | Body weight at end of period. | Nitrogen intake. | Urine. | | | | Remarks. |
|---|---|---|---|---|---|---|---|
| | | | Total nitrogen. | Total creatinine nitrogen. | Uric acid nitrogen. | Allantoin nitrogen. | |
| Rat 46. ♀. Initial weight, 62 gm. | | | | | | | |
| *days* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| 1–16 | 90 | 1.95 | 0.64 | 18.7 | 2.3 | 69.3 | Diet 7 (whole casein) throughout. |
| 17–32 | 108 | 2.13 | 1.08 | 27.1 | 2.8 | 74.4 | |
| 33–48 | 129 | 2.52 | 1.41 | 31.1 | 3.6 | 89.7 | |
| 49–64 | 152 | 2.89 | 1.50 | 35.6 | 3.8 | 92.4 | |
| 65–80 | 171 | 2.69 | 2.09 | 43.8 | 5.0 | 115.8 | |
| 81–96 | 175 | 2.41 | 1.63 | 52.0 | 4.8 | 90.8 | |
| 97–112 | 186 | 2.56 | 1.81 | 55.2 | 5.3 | 105.6 | |
| 113–128 | 190 | 2.15 | 1.71 | 57.2 | 6.5 | 106.7 | |
| Rat 49. ♀. Initial weight, 57 gm. | | | | | | | |
| 1–16 | 75 | 1.80 | 0.68 | 16.6 | 2.2 | 72.0 | Diet 7 (whole casein) throughout. |
| 17–32 | 98 | 2.06 | 1.15 | 24.9 | 2.9 | 80.2 | |
| 33–48 | 122 | 2.58 | 1.16 | 27.8 | 4.0 | 89.0 | |
| 49–64 | 147 | 3.15 | 1.40 | 33.9 | 4.6 | 86.7 | |
| 65–80 | 168 | 3.12 | 2.08 | 42.9 | 5.4 | 109.4 | |
| 81–96 | 174 | 2.43 | 1.55 | 50.2 | 5.6 | 102.9 | |
| 97–112 | 186 | 2.58 | 1.81 | 58.8 | 5.9 | 95.7 | |
| 113–128 | 194 | 2.65 | 1.80 | 57.0 | 6.7 | 93.1 | |

225 cc. each of the final filtrates were employed for the titrations with ammonium thiocyanate. Analysis of human and rat urines to which allantoin had been added showed recoveries of 85 to 95 per cent. This is about the usual degree of accuracy observed by others with the Wiechowski-Handovsky method.

The results of the experiments are shown in Tables II to VII inclusive. In most of the experiments the analytical data are

tabulated in periods of 16 days each, although the analyses were made at 8 day intervals. Inasmuch as the quantitative collection of urine in small animals is inherently difficult, we felt that long periods were necessary in order to yield convincing results.

TABLE III.

*Nitrogen Metabolism on Diets of Completely Hydrolyzed Casein.*

| Period | Body weight at end of period. | Nitrogen intake. | Urine. | | | | Remarks. |
|---|---|---|---|---|---|---|---|
| | | | Total nitrogen. | Total creatinine nitrogen. | Uric acid nitrogen. | Allantoin nitrogen. | |
| Rat 45. ♀. Initial weight, 74 gm. | | | | | | | |
| *days* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| 1–16 | 107 | 2.43 | 0.87 | 22.1 | 3.2 | 84.4 | Diet 7 (whole casein). |
| 17–32 | 117 | 1.69 | 0.92 | 36.2 | 4.5 | 87.5 | Diet 12 (completely hydrolyzed casein). |
| 33–48 | 123 | 1.60 | 1.02 | 37.1 | 4.5 | 95.4 | |
| 49–64 | 136 | 2.04 | 1.25 | 44.7 | 5.1 | 94.4 | |
| 65–80 | 142 | 1.74 | 1.18 | 50.4 | 5.1 | 100.8 | |
| 81–96 | 153 | 1.71 | 1.13 | 55.9 | 5.3 | 93.8 | |
| 97–112 | 158 | 1.83 | 1.25 | 59.7 | 6.0 | 95.0 | |
| 113–128 | 166 | 1.85 | 1.21 | 63.2 | 6.5 | 100.7 | |
| Rat 57. ♀. Initial weight, 90 gm. | | | | | | | |
| 1–8* | 111 | 1.61* | 0.66* | 14.8* | 2.2* | 42.8* | Diet 7 (whole casein). |
| 9–24 | 114 | 1.87 | 1.09 | 41.8 | 4.7 | 87.3 | Diet 12 (completely hydrolyzed casein). |
| 25–40 | 121 | 1.53 | 1.03 | 37.0 | 4.0 | 84.8 | |
| 41–56 | 134 | 1.83 | 1.15 | 40.5 | 4.7 | 91.3 | |
| 57–72 | 136 | 1.57 | 1.15 | 50.4 | 5.0 | 103.0 | |
| 73–88 | 140 | 1.32 | 1.00 | 52.9 | 5.0 | 82.3 | |
| 89–104 | 148 | 1.60 | 1.16 | 56.9 | 5.7 | 93.1 | |
| 105–120 | 147 | 1.30 | 1.13 | 53.3 | 6.4 | 93.0 | |

* The first period in this experiment lasted for 8 instead of 16 days. Hence the low analytical figures.

In Table VII the analytical data are shown in periods of 8 days each. This was done because the two experiments therein recorded were of approximately half the duration of those presented in Tables II to VI inclusive.

Despite the difficulties in collection, and the unsatisfactory

nature of the Wiechowski-Handovsky procedure for allantoin determinations, the results of the experiments are quite consistent. As was to be expected, greater irregularity was observed in the allantoin figures than in the other urinary components. This is undoubtedly to be attributed to errors inherent in the

TABLE IV.

*Nitrogen Metabolism on Diets of "Deficient Digest."*

| Period. | Body weight at end of period. | Nitrogen intake. | Urine. | | | | Remarks. |
|---|---|---|---|---|---|---|---|
| | | | Total nitrogen. | Total creatinine nitrogen. | Uric acid nitrogen. | Allantoin nitrogen. | |
| | | | Rat 55. ♂. Initial weight, 85 gm. | | | | |
| *days* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| 1–16 | 130 | 3.02 | 1.27 | 29.1 | 4.0 | 89.5 | Diet 7 (whole casein). |
| 17–32 | 109 | 1.51 | 1.31 | 40.3 | 3.4 | 60.4 | Diet 11 ("deficient digest"). |
| 33–48 | 107 | 1.62 | 1.31 | 37.4 | 3.1 | 63.7 | |
| 49–64 | 99 | 1.62 | 1.26 | 31.6 | 3.3 | 64.7 | |
| 65–80 | 96 | 1.34 | 1.07 | 37.3 | 3.0 | 43.5 | |
| 81–96 | 92 | 1.14 | 0.95 | 31.3 | 2.8 | 44.6 | |
| 97–112 | 87 | 1.12 | 0.97 | 30.9 | 3.3 | 55.1 | |
| | | | Rat 56. ♀. Initial weight, 90 gm. | | | | |
| 1–16 | 131 | 3.02 | 1.19 | 32.6 | 4.3 | 93.0 | Diet 7 (whole casein). |
| 17–32 | 111 | 2.01 | 1.23 | 40.3 | 3.6 | 70.0 | Diet 11 ("deficient digest"). |
| 33–48 | 108 | 1.89 | 1.44 | 41.4 | 3.8 | 74.6 | |
| 49–64 | 104 | 1.67 | 1.37 | 37.4 | 3.8 | 62.3 | |
| 65–80 | 99 | 1.39 | 1.20 | 40.2 | 3.8 | 57.0 | |
| 81–96 | 94 | 1.12 | 1.04 | 34.6 | 3.5 | 46.0 | |
| 97–112 | 91 | 1.19 | 1.11 | 33.7 | 3.5 | 53.2 | |

allantoin method. On the diet of whole casein (Table II) the animals gained in weight at normal rates, and the output of all urinary components steadily rose. The figures for total creatinine and uric acid show increases during the course of the experiments of approximately 300 per cent. The allantoin elimination, though irregular, increased 30 to 50 per cent. Similar results were secured with the diet of completely hydrolyzed

casein (Table III), but inasmuch as the animals upon this diet did not grow quite as rapidly as those on the whole casein ration, the increments in urinary components were not quite so large.

The results of the experiments with the "deficient digest" (Table IV) are in striking contrast to those with whole or hydro-

TABLE V.

*Nitrogen Metabolism on Diets of "Deficient Digest" Plus Arginine.*

| Period. | Body weight at end of period. | Nitrogen intake. | Urine. | | | | Remarks. |
|---|---|---|---|---|---|---|---|
| | | | Total nitrogen. | Total creatinine nitrogen. | Uric acid nitrogen. | Allantoin nitrogen. | |
| Rat 44. ♂. Initial weight, 71 gm. | | | | | | | |
| *days* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| 1–16 | 104 | 2.36 | 0.78 | 19.0 | 2.2 | 74.9 | Diet 7 (whole casein). |
| 17–32 | 90 | 1.88 | 1.28 | 41.0 | 2.8 | 59.5 | Diet 11 ("deficient digest") plus arginine. |
| 33–48 | 87 | 1.63 | 1.25 | 35.5 | 2.5 | 52.2 | |
| 49–64 | 82 | 1.65 | 1.36 | 33.3 | 3.2 | 55.4 | |
| 65–80 | 81 | 1.48 | 1.35 | 30.4 | 2.7 | 56.3 | |
| 81–96 | 77 | 0.92 | 0.90 | 28.4 | 2.3 | 40.0 | |
| 97–112 | 74 | 1.03 | 1.05 | 27.6 | 2.4 | 56.2 | |
| 113–128 | 70 | 0.88 | 0.85 | 25.0 | 2.4 | 53.9 | |
| Rat 51. ♂. Initial weight, 69 gm. | | | | | | | |
| 1–16 | 102 | 2.52 | 0.85 | 19.9 | 2.7 | 92.5 | Diet 7 (whole casein). |
| 17–32 | 92 | 1.93 | 1.30 | 36.7 | 3.3 | 52.2 | Diet 11 ("deficient digest") plus arginine. |
| 33–48 | 89 | 1.69 | 1.26 | 33.0 | 3.2 | 40.0 | |
| 49–64 | 87 | 1.82 | 1.39 | 34.1 | 3.3 | 48.6 | |
| 65–80 | 84 | 1.54 | 1.19 | 30.3 | 3.0 | 50.2 | |
| 81–96 | 82 | 1.09 | 0.99 | 30.8 | 2.6 | 46.7 | |
| 97–112 | 80 | 1.13 | 1.00 | 28.7 | 2.8 | 51.3 | |
| 113–128 | 78 | 1.05 | 0.97 | 25.3 | 2.9 | 53.1 | |

lyzed casein. As will be observed, the values for allantoin, instead of increasing, promptly diminished 40 to 50 per cent following the change from the casein to the deficient ration. In each experiment the most pronounced decrease was observed in the first period following the dietary alteration, but further though somewhat variable decreases occurred in subsequent

periods. The uric acid elimination also diminished, but the variations from the normal values are not quite so striking as in the case of allantoin. Total creatinine manifested a moderate increase followed by a decline, but at no time did the elimination on the inadequate diet fall below the output on the adequate ration.

TABLE VI.

*Nitrogen Metabolism on Diets of "Deficient Digest" Plus Histidine.*

| Period. | Body weight at end of period. | Nitrogen intake. | Urine. | | | | Remarks. |
|---|---|---|---|---|---|---|---|
| | | | Total nitrogen. | Total creatinine nitrogen. | Uric acid nitrogen. | Allantoin nitrogen. | |
| Rat 48. ♀. Initial weight, 69 gm. | | | | | | | |
| *days* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| 1–16 | 100 | 2.47 | 0.90 | 20.0 | 2.2 | 79.1 | Diet 7 (whole casein). |
| 17–32 | 118 | 2.51 | 1.15 | 29.5 | 3.1 | 78.9 | Diet 11 ("deficient digest") plus histidine. |
| 33–48 | 121 | 2.23 | 1.27 | 33.8 | 3.6 | 95.3 | |
| 49–64 | 139 | 2.83 | 1.53 | 40.4 | 4.0 | 90.3 | |
| 65–80 | 154 | 2.51 | 1.60 | 46.4 | 4.6 | 90.4 | |
| 81–96 | 163 | 2.35 | 1.31 | 62.3 | 5.3 | 94.2 | |
| 97–112 | 172 | 2.91 | 1.66 | 61.2 | 5.3 | 93.1 | |
| 113–128 | 179 | 2.69 | 1.66 | 60.2 | 6.4 | 105.6 | |
| Rat 52. ♀. Initial weight, 47 gm. | | | | | | | |
| 1–16 | 78 | 1.84 | 0.54 | 15.5 | 2.0 | 63.0 | Diet 7 (whole casein). |
| 17–32 | 91 | 1.79 | 0.84 | 22.7 | 3.1 | 53.8 | Diet 11 ("deficient digest") plus histidine. |
| 33–48 | 106 | 1.81 | 0.97 | 27.7 | 2.9 | 70.1 | |
| 49–64 | 126 | 2.27 | 1.27 | 31.2 | 4.1 | 87.3 | |
| 65–80 | 138 | 1.97 | 1.19 | 42.5 | 4.5 | 89.6 | |
| 81–96 | 145 | 1.71 | 1.08 | 56.0 | 4.7 | 80.2 | |
| 97–112 | 145 | 2.03 | 1.22 | 55.1 | 4.0 | 88.7 | |
| 113–128 | 154 | 1.75 | 1.28 | 55.1 | 5.2 | 93.8 | |

As is shown in Table V, the addition of arginine to the deficient diet failed entirely to induce growth, or to influence the output of any of the urinary components. Indeed, the experimental data obtained from the two arginine-fed rats are in every respect similar to those secured with the "deficient digest" animals.

It appears that the addition of arginine, under the conditions of our experiments, exerted no influence upon either purine or creatine-creatinine metabolism.

In Table VI are presented the results of two experiments upon the effects of including histidine in the ration. As will be seen,

TABLE VII.

*Nitrogen Metabolism on Diets Deficient in Tryptophane.*

| Period. | Body weight at end of period. | Nitrogen intake. | Urine. | | | | Remarks. |
|---|---|---|---|---|---|---|---|
| | | | Total nitrogen. | Total creatinine nitrogen. | Uric acid nitrogen. | Allantoin nitrogen. | |
| Rat 123. ♀. Initial weight, 72 gm. | | | | | | | |
| *days* | *gm.* | *gm.* | *gm.* | *mg.* | *mg.* | *mg.* | |
| 1–8 | 88 | 0.93 | 0.45 | 9.8 | 1.7 | 25.5 | Diet 7 (whole casein). |
| 9–16 | 81 | 0.61 | 0.36 | 12.3 | 2.0 | 27.7 | Diet 12 minus tryptophane. |
| 17–24 | 78 | 0.45 | 0.42 | 16.2 | 2.0 | 31.2 | |
| 25–32 | 76 | 0.52 | 0.44 | 17.9 | 1.9 | 27.2 | |
| 33–40 | 76 | 0.44 | 0.26 | 14.8 | 1.9 | 25.7 | |
| 41–48 | 73 | 0.42 | 0.34 | 14.3 | 2.2 | 31.9 | |
| 49–56 | 73 | 0.42 | 0.35 | 12.7 | 1.8 | 30.4 | |
| Rat 124. ♀. Initial weight, 109 gm. | | | | | | | |
| 1–8 | 131 | 1.43 | 0.64 | 16.1 | 2.4 | 41.2 | Diet 7 (whole casein). |
| 9–16 | 121 | 0.80 | 0.59 | 24.2 | 2.9 | 38.4 | Diet 12 minus tryptophane. |
| 17–24 | 116 | 0.52 | 0.50 | 19.2 | 2.2 | 43.9 | |
| 25–32 | 114 | 0.82 | 0.58 | 21.1 | 2.5 | 37.3 | |
| 33–40 | 114 | 0.68 | 0.57 | 19.4 | 2.5 | 45.9 | |
| 41–48 | 112 | 0.63 | 0.55 | 19.5 | 2.7 | 40.3 | |
| 49–56 | 110 | 0.61 | 0.52 | 17.6 | 2.4 | 35.0 | |

the addition of this amino acid to Diet 11 not only permitted growth, but led to regular increases in the excretion of total creatinine, uric acid, and allantoin. Furthermore, the increments in output of the urinary components are of the same order as in animals upon the whole casein diet. Obviously, histidine and arginine bear quite different relations to purine metabolism.

Following the general procedure of Ackroyd and Hopkins we

have performed two experiments involving tryptophane deficiency. The experiments were begun after the others were well under way, and are of shorter duration than those which involved histidine deficiency. The results are shown in Table VII. Although the percentage losses in weight were almost as great, for equal periods of time, as in the rats deprived of histidine, the values for allantoin and uric acid excretion remained quite constant throughout.

## DISCUSSION.

The data outlined above are of interest from several points of view. First, they appear to indicate that histidine is the precursor of tissue purines. In this respect our results are in accord with those of Ackroyd and Hopkins. We believe that this deduction is justified despite the irregularities in the figures for allantoin excretion. The unsatisfactory nature of the Wiechowski-Handovsky method is recognized by all who have employed it, and the undesirable features are emphasized when the method is applied to dilute urines, such as are obtained in metabolism studies upon rats. That the fluctuations in some of our allantoin data are to be attributed to errors inherent in the method of analysis, and not to incompleteness in urine collection, is indicated by the values for total creatinine and uric acid. The latter are quite regular in all of the experiments. Where the diet permitted growth, the increases in output of total creatinine are roughly proportional to the increments in body weight of the animals. We do not attach much significance to the *absolute quantities of allantoin* obtained either in our or in any other experiments upon rats hitherto reported in the literature, but we believe that the *relative increases* observed uniformly when histidine is present in the diet, and the *relative decreases* seen invariably when the amino acid is excluded from the ration, justify the conclusion that histidine and allantoin are closely related to each other in metabolism.

Furthermore, we have reached this conclusion somewhat contrary to our earlier ideas. During the progress of the experiments, it seemed to us not unlikely that the differences in the effects of histidine and arginine upon purine metabolism might

be due solely to the fact that the addition of one amino acid permitted growth, while the inclusion of the other did not. In other words, it seemed probable to us that the alterations in urine composition were the result of, and secondary to, the ability or inability of the rats to increase in body weight, and did not indicate necessarily a precursor relationship between histidine and body purines. We were inclined to the opinion that with loss in weight, and the resulting decrease in mass of active protoplasm, there might occur a diminution in the output of all endogenous products. In order to test this possibility, it was evidently necessary to determine the effects upon urinary composition of another type of dietary deficiency. Such an experiment appeared to be capable of serving as a crucial test for a possible relationship between histidine and purines. With these considerations in mind, we conducted the experiments upon the influence of excluding tryptophane from the diet. The results were striking. Instead of obtaining falls in allantoin and uric acid as we had anticipated, the values for both of these substances remained quite constant throughout the experiments. On the other hand, the total creatinine behaved just as it did during histidine deficieny, showing a tendency to increase at first, followed later by a decrease, but at no time falling below the original level observed on the adequate diet. It is true that the experiments upon tryptophane deficiency were only half as long as those which involved a deficiency of histidine; but in the latter, very pronounced decreases in allantoin excretion occurred during the first periods following the change to the inadequate ration. The conclusion appears to be warranted, therefore, that *under ordinary conditions of diet, histidine is a mother-substance of allantoin.* If this conclusion is correct, it is evident that one reason why histidine is an indispensable component of the diet is that it is required for nuclear synthesis.

In this connection it should be noted that our results are not necessarily in conflict with those of Abderhalden and Einbeck (1909), and Abderhalden, Einbeck, and Schmid (1910). These authors report that the feeding of histidine to fasting dogs, or the addition of histidine to the diet of a well fed animal, fails entirely to influence the allantoin output. We have discussed this question, and the literature bearing upon it, in a former

communication (Rose, 1921). Ackroyd and Hopkins (1916) also properly raise the question as to whether an abnormal condition like fasting affords the best opportunity for investigating the fate of an amino acid. In regard to the addition of excess histidine to an already adequate diet the same authors make the following interesting comment; "When an animal is in a state of full nutrition it does not follow that such a process as the synthesis of the purine ring would necessarily be much accelerated or increased by mere increase in the supply of its raw material." As we have stated on several occasions elsewhere (Rose, 1921, 1923), it does not seem unreasonable to suppose that the synthesis of a tissue component may be limited quantitatively to the anabolic needs of the organism for that particular ingredient. If this conception is correct, the addition of histidine to an already adequate diet should not necessarily lead to an increase in allantoin excretion.

In contrast to the observations of Ackroyd and Hopkins our experiments show quite clearly that arginine cannot replace histidine in purine synthesis. This result serves to emphasize and reenforce the conclusion of Rose and Cox (1924), that in the absence of histidine from the diet its functions cannot be assumed vicariously by arginine. It appears evident that neither in growth nor in purine metabolism can the two diamino acids be regarded as mutually interchangeable.

Under the conditions which pertained in our experiments, no relationship was observed between the arginine content of the diet and the total creatinine elimination in the urine. The information in the literature concerning the relationship of arginine to creatine-creatinine metabolism is quite conflicting.[2] Thompson (1917), and Gross and Steenbock (1921) expressed the opinion that increased arginine consumption leads to an exaggerated creatine production. Somewhat similar conclusions had been reached at an earlier date by McCollum and Steenbock (1912–13), and others. On the contrary, Myers and Fine (1915), Baumann and Marker (1915), and Baumann and Hines (1918) were unable to observe any relationship between the arginine content of the food and the creatine or creatinine output in the

[2] For a review of the literature, see the paper of Hunter (1922).

excreta. As we have stated elsewhere (Rose and Cox, 1924), it is quite likely that the Kossel-Kutscher method of precipitating the diamino acids does not completely remove arginine. Assuming, therefore, that this amino acid is the precursor of creatine, it is possible that even on the deficient diets our animals may have received adequate amounts for creatine anabolism, and that quantities furnished in excess of the anabolic needs were catabolized by more direct methods. In order to answer definitely the question of a possible relationship between arginine and creatine-creatinine metabolism, it would appear necessary to employ a diet known to be absolutely devoid of the amino acid in question. We are endeavoring to prepare such a material at the present time.

## SUMMARY.

1. Studies have been made of creatine and purine metabolism in growing rats upon diets in which the nitrogen was supplied, respectively, in the form of casein, completely hydrolyzed casein, and hydrolyzed casein from which histidine and arginine had been precipitated by the Kossel-Kutscher procedure. The diets of whole casein and of completely hydrolyzed casein led to the excretion of progressively increasing quantities of total creatinine (creatine plus creatinine), allantoin, and uric acid, which were roughly proportional to the increments in body weight of the animals. On diets of hydrolyzed casein from which histidine and arginine had been precipitated, the output of allantoin decreased 40 to 50 per cent. The uric acid excretion also decreased, but the variations from the normal values were not quite so striking as in the case of allantoin. Total creatinine manifested an increase followed by a decline, but at no time did the output fall below the level seen on the adequate ration.

2. The effects upon purine metabolism are not to be attributed solely to the losses in weight of the rats on the deficient diets as is indicated by the results of experiments in which a deficiency of tryptophane was induced. When the ration was adequate in every respect except as regards its tryptophane content, the animals steadily declined in weight, but the output of allantoin and uric acid remained quite constant.

3. The addition of histidine to diets in which the component hydrolyzed casein had previously been subjected to silver precipitation, led to increases in the excretion of total creatinine, uric acid, and allantoin, until the quantities eliminated were of the same order as in animals upon whole casein. On the contrary, the addition of arginine to the deficient rations failed entirely to affect the output of any of the urinary components. It is evident from these investigations that *arginine and histidine are not interchangeable in purine metabolism.* Our data also appear to indicate that *histidine is one of the precursors of purines.*

4. Under the conditions which pertained in our experiments, *no relationship was observed between the arginine content of the diet and the total creatinine elimination in the urine.* This may have been due to the fact that none of our diets were completely devoid of arginine.

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# ELECTROSTATIC FORCES IN THE DIFFUSION OF WATER THROUGH COLLODION MEMBRANES BETWEEN SOLUTIONS OF MIXED ELECTROLYTES.

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## I.

## INTRODUCTION.

These experiments were performed to find what rôle electrostatic diffusion may possibly play in determining the transfer of water between solutions of the kinds found in living organisms. It was first necessary to repeat many experiments, done by previous investigators of diffusion phenomena, upon the transport of water through membranes into solutions each containing a single salt. Then solutions containing mixtures of electrolytes were studied, and principles were formulated for predicting the rates of diffusion of water into and between such solutions. Finally it was demonstrated that electrostatic diffusion potentials are very small in solutions containing large proportions of neutral univalent electrolytes.

The rate at which water, either pure or as a solvent, travels by diffusion, can be studied directly only in systems divided by membranes. The influence of the membrane upon such diffusion was therefore naturally neglected in the early demonstrations of water diffusion, such as that of Nollet (1). In the classical work of Dutrochet (2) attention was drawn to the different influences of diverse kinds of membranes, and their rôle was discussed by contemporary investigators such as Fischer (3), Poisson (4), and Magnus (5). Later Brücke (6), Vierordt (7), and Traube (8) compared several different kinds of natural and artificial membranes, but without furnishing quantitative results.

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Quantitative accounts of diffusion processes begin from the studies of solute diffusion by Graham (9), for on their basis Fick (10) was able to formulate his "law" of diffusion. The meaning of this mathematical description became vaguely realized with the study of osmotic pressure by Pfeffer (11) and its interpretation by van't Hoff (12). In one respect these investigations led to neglect; they emphasized the rôle of the solute, whereas the more important thing in both osmotic pressure and diffusion phenomena would seem to be the solvent.

Although osmotic pressure accounted for the movements of the solvent in most cases, it was early recalled that another force was often responsible for the migration of liquids through membranes. This was the electrical force, and although its influence upon water transport had been observed as early as 1808 by Reuss (13), its influence was first quantitatively investigated by Wiedemann (14), and later by Quincke (15) and Lamb (16). On the basis of this knowledge it was demonstrated by Helmholtz (17), Lenard (18), Flusin (19), Perrin (20), Nolan (21), McTaggart (22), and others that potentials sufficient to be accompanied by diffusion are present in many heterogeneous systems. These diffusion potentials are electrostatic charges, due to the presence of ions or other unequally distributed forces. Coehn (23) was able to show that the charges were quantitatively related to the dielectric value of the solvent. The actual electrostatic forces have been evaluated in terms of diffusion rates through membranes by Flusin (24), Perrin (25), Girard (26), Bartell (27), and Loeb (28). Electrostatic diffusion has been further illustrated and discussed in the work of Hamburger (29), Bernstein (30), Bethe and Toropoff (31), Freundlich (32), T. Hamburger (33), Loeb (34), and Umezawa (35).

The upshot of this slowly evolved study of an unexpectedly complicated physical phenomenon is that (*a*) the membrane plays no rôle in osmotic diffusion except that it usually delays the spread of solute more than that of solvent; and (*b*) the membrane plays an important rôle in electrostatic diffusion in localizing at its surfaces potential charges which depend upon net differences between the solutes on the two sides of it.

Considerable confusion exists as to the importance of the electrostatic diffusion of water under the conditions found in living organisms. To resolve this confusion it is necessary to know what will occur in characteristic solutions containing many solutes in systems outside organisms. It will be shown below that in the presence of physiological solutions only very weak electrostatic diffusions occur.

XUM

II.

*Method.*

The methods which have been used in the past in diffusion studies have been on the whole very cumbersome. Rapid results can be quickly and easily obtained only when there are large surfaces of contact between small volumes of two liquids whose changes of volume can be measured. Sac-like collodion membranes connected to manometer tubes, such as were devised by Lillie (36) and used extensively by Loeb (34), were, after some trial of other materials, found to answer the requirements; and our experience is that nothing can compensate for the rapidity and ease of this technique for observing the migration of the solvent. Such membranes are only relatively semipermeable, but the diffusion of water under the conditions studied is much more rapid than that of solutes, so that accurate comparative values can be observed. We describe our methods in detail because only after performing hundreds of experiments were we able to make our procedure consistent and to evaluate the results properly.

Collodion membranes were prepared from U. S. P. collodion solutions in either of two ways. By method *A*, about 15 cc. of collodion solution were poured into a 50 cc. Erlenmeyer flask which had been rinsed with alcohol. The solution was rapidly poured out again, rotating the flask by hand in the inverted position, and the rotation was continued for 5 minutes. Then the flask was allowed to stand inverted but with its mouth unobstructed for 5 minutes to 5 hours, but usually for 15 minutes. The sac-like membrane was separated from the flask by running some water under the membrane and by pulling gently upon it. Afterwards it was kept in tap water. In method *B*, similar sacs were made by the exact procedure described by Loeb (37), allowing the filled flask to drain of collodion solution for 2 minutes and to dry for 2 minutes, and then hardening the membrane in running water for 5 minutes before separating it from the flask.

The collodion membranes used varied considerably in porosity, presumably due to variations in their preparation, even when prepared as identically as possible. On the whole, those prepared by method *A* were less penetrable by large molecules. That some of the membranes were relatively very impenetrable was indicated by the fact that hemoglobin solutions failed both to stain them and to pass through them, both of which depend upon the ability of the hemoglobin molecules to enter the pores of the membranes. Most of the membranes, however, were penetrated by hemoglobin. The rate at which water under hydrostatic pressure passed through a particular membrane did not vary during many days after its manufacture, and the rate of penetration into M/4 sucrose solutions was not found to change from the time of manufacture because of age or subsequent treatment. The relation between average pore size and diffusion through collodion membranes has been worked out by Bartell and Carpenter (38).

For use the 50 cc. sacs were tied with rubber bands to perforated rubber stoppers. The sacs were filled with solution and glass tubes with an in-

ternal diameter of 3 mm. were passed through their stoppers to serve as manometers. The flask-shaped sacs were each immersed in 500 cc. of water in 600 cc. beakers, each sac having been filled to such an extent that after immersion the level in the manometer would be 20 to 35 mm. above the level of the solution outside. In every case the stoppers were well below the surface of the outside solution, and the sacs did not touch any surface of the beakers. Changes of level in the 3 mm. tubes indicated continuously the rates of passage of water in either direction through the membranes, so long as the hydrostatic pressures in the inner solutions were greater by more than 8 mm. than those in the outer vessels. With positive pressures less than 8 mm. the collodion sacs began to collapse inward. The amount of each change in level, which indicated the change in volume of the inner solution except for the slight stretching of the sac, was read at intervals as the difference between the initial and existing water levels.

The method which one chooses for interpreting the numerical readings makes a considerable difference in the conclusions reached, and the choice must be made with due regard to the meaning of the experimental procedure. First, one always refers the values obtained to the initial concentrations of the solutes. The changes of concentration within a few minutes do not sensibly affect the values obtained, but to minimize this error, only the first 5 minutes of a test with a 50 cc. sac can be relied upon. Second, one is actually observing changes in water level in the inner container, and only by taking a sufficiently short time interval are the values free from the influence of hydrostatic pressure. If the time courses of various experiments are plotted, it can be seen how diverse are the influences of these two factors in different cases. We attempted to use as a measure of diffusion rate the strength of sucrose solution, or of electrical potential, or of hydrostatic pressure required to oppose the inflow of water. But in all cases the measurements were entirely uncertain even after the very great trouble of carrying them out; in fact many of the diffusion forces are so great that they cannot be opposed by any concentration of osmotically active substances, and yet they are opposed by very small hydrostatic pressures. Hydrostatic pressures cannot be evaluated by comparison with other tensions as regards their influences upon the transport of water through these membranes.

In every diffusion experiment there are initial adjustments, usually included within the 1st minute, which are not manifestations of the forces being studied. All our measurements of diffusion

rates are, in accordance with the above considerations, what we may picture as tangents to the greatest slope of the plotted time curves. At first we determined this value by plotting the data to simulate a kymographic record of the changes in the manometer levels; later we found that ordinarily the value so obtained is equivalent to the rate of manometer change just after the 1st minute of the experiment has elapsed, so that in most cases the mean of the rates for the 2nd and 3rd minutes has been used. All the values have thus been arbitrarily expressed as mm. rise of water level per minute in a 3 mm. tube. Actually a rise of 1 mm. in the tube represents an influx of 8 c. mm., or 0.12 c. mm. of water per sq. cm. of membrane. This method of evaluation gave results considerably more consistent than any other, and turned out to have the very important advantage of requiring only 3 or 4 minutes for each test.

The numerical results require further limitation in their interpretation, chiefly because of the variability of the membranes. With no one membrane were we able to get duplicate results on separate days which regularly agreed within 10 per cent, and with different membranes the results were still less comparable, the chief variables being absolute ones. The most reliable results were obtained by using the same membrane for all the tests of a series, using it in succession for those points which were to be compared most closely. By this procedure, and because the chief errors are absolute ones, the relative errors were less than 10 per cent in the cases, which were the chief ones studied, where diffusion was rapid.

III.

*Diffusion of Water through Plain Collodion Membranes.*

The simplest conditions for diffusion are those where pure solvent and solution are allowed to interchange without the intervention of a membrane. The technical difficulties of making observations upon the solvent under these conditions have proved insuperable, and for this reason we do not know how water diffuses in the absence of a membrane. But since the results in membrane systems containing only non-electrolytes are regularly predictable from Fick's "law," it is usually believed that the

membrane does not complicate the diffusion of water in such systems. In the presence of electrolytes, however, all the intricate phenomena known to complicate the measurements of electrical conductivity and osmotic pressure are at work, and the only approach to measuring the diffusion rate for the solvent in the absence of a membrane is by the calculation of an "error" in the comparative measurements of the transport numbers, electrical conductivities, and diffusion rates of various solutes. Such a procedure is suggested by the results of Nernst (39), Abegg and Gaus (40), Svedberg (41), and others.

To lead up to the experiments which we have done upon the diffusion of water into and between solutions of many electrolytes, it is necessary to review briefly what we know of diffusion through membranes into and out of solutions of single salts. In order that we might understand thoroughly the results of previous investigators, particularly of Girard, Bartell, and Loeb, we have repeated and confirmed very many of their observations.

Collodion membranes are typical of many artificial membranes whose properties are commonly supposed to depend upon the sizes of the pores (Tinker (42), Bartell and Carpenter (38)). The size is not correlated as a mechanical sieve, however, but as a localizer of electrostatic charges. If the pores are either too large or too small these charges are immeasurably small; and only with membranes of intermediate pore size are the influences upon diffusion important. For this reason the plain collodion membranes made by our method *A* showed very little influence upon the diffusion of water through them. Only in the presence of considerable concentrations of sodium sulfate or of potassium ferrocyanide were marked electrostatic effects upon diffusion found with them; in all other cases tried the membranes were passive, whether the solutions were placed inside the sacs and pure water outside, or whether the water was placed inside under an initial hydrostatic pressure of 150 mm. or more. From this experience one must realize that diffusions of this sort are not so readily obtained as one is led to believe from many published investigations; mention of the peculiar conditions which must be satisfied is generally omitted.

When the collodion membranes were made by method *B*, the results described for plain collodion membranes by Loeb (34)

and Bartell and Carpenter (43) were found to hold true, though we never succeeded in preparing plain membranes (about 70 were tested) which gave as rapid diffusion as did Loeb's membranes.

These results were briefly as follows: (*a*) Non-electrolytes are without measurable influence upon diffusion of water except in concentrated solutions. Their influence is a function only of the osmotic pressure of their solutions. This we found true for sucrose, dextrose, ethyl alcohol, glycerol, and urea. The three latter substances penetrate the membranes so rapidly that measureable diffusion rates are not exhibited in concentrations less than M/4. (*b*) Cations are without predominating influence upon diffusion, no matter what their valence or what the hydrogen ion concentrations of their solutions. The absence of cation influence shows that the usual negative electrostatic charge of the plain collodion membrane compared to that of the water cannot be reversed by solutes, as Loeb (44) was able to demonstrate. But that cations have some influence upon the forces at the membrane is shown by the decrease in the relative influence of multivalent anions in moderately strong concentrations. (*c*) Anions have only a small predominating influence when univalent. Bivalent (sulfate), trivalent (ferrocyanide, citrate), and quadrivalent (ferrocyanide) ions influence the diffusion rate of water very markedly. The comparative magnitudes have been determined by Loeb (45), and need not be repeated here. Loeb (44, 46) has also shown that the influence of multivalent ions is almost constant for hydrogen ion concentrations from pH 2 to 12. It is probable that ions of the same valence have slightly different electrostatic values, though no consistent differences were found. In general the influence of the anions increases with their valence.

It is important to differentiate strictly between membranes which have been treated with a protein and those untreated. This was not done in the earlier work of Loeb, because he began his investigations with treated membranes, and by this means later realized the remarkable influence of such treatment upon the diffusion of water. In his very latest papers Loeb (46) differentiated emphatically, at least qualitatively, between treated and untreated membranes; and in the latter obtained influences upon diffusion only by anions, as we did.

The rôle of the membrane in the diffusion of water has been

shown by Dutrochet (2), Flusin (19), Girard (26), Bartell (27), and others to be independent of its composition, providing dissolved electrolytes are not contained in it. Thus, similar results have been obtained with cracked glass, rubber, porcelain, cupric ferrocyanide, and other metallic precipitates.

IV.

*Diffusion of Water into Simple Solutions through Collodion Membranes Treated with Proteins.*

It was discovered by Loeb (47) that collodion membranes which had been immersed in an aqueous solution of a protein were profoundly modified as regards the passage of water through them. The modification was exhibited, however, only in the presence of certain concentrations of certain electrolytes. The influence of the membranes and of the salts are briefly summarized below.

*1. Influence of Proteins in Membranes.*—The properties of the membranes upon which the peculiarities of diffusion depend we have studied by varying their preparation in several ways. For most of the experiments the treatment with proteins was that used by Loeb (28). 50 cc. collodion sacs, made by both method *A* and method *B*, were filled with fresh protein solutions such as 1 or 2 per cent Knox's gelatin or 10 per cent hemolyzed beef corpuscles, and allowed to stand for 1 to 48 hours at room temperature. Some of the protein molecules were retained in the membrane, and remained, at least in part, for an indefinite time and irrespective of subsequent experimental treatment. Slowly the influence of the protein was reduced in amount, both by use and time, as though the protein very gradually diffused away from the pores of the membrane and was lost in the liquid. A coating of agar did not alter the properties of the membrane as regards diffusion.

The presence of hemoglobin was always indicated by the red color conferred on the membrane; the presence of gelatin could often be ascertained by immersing the membrane in a solution of ferric chloride, when it was stained a permanent brown color. This staining did not alter its properties as regards diffusion. In a few cases membranes did not show these colors after a brief immersion in the protein, and they also failed to manifest the

diffusion behavior characteristic of other treated ones. Every membrane treated with a protein should be tested for the success of the treatment, by measuring the rate of diffusion into a standard solution of some triunivalent salt or the like.

That the protein was actually in the pores of the membrane was proved in the following way. If previously immersed in the protein solution on the outside only, instead of on the inside, there was no difference in the direction or intensity of water migration. If treated on both sides, no diversity resulted; nor, if treated with gelatin on one side and hemoglobin on the other side. There was, therefore, no condition under which the spatial relation of protein to collodion could be varied. Moreover, once treated with a protein, no difference in behavior of sacs made by method *A* and by method *B* could be noted.

That the protein must have been in solution was shown by the following tests. The alcohol-soluble proteins, zein and gliadin, were successfully dissolved in some collodion solution, and this made into membranes by method *B*. No influence of cations upon the migration of water was then exhibited. When a gelatin-treated membrane was boiled in water, its properties were unchanged; but if treated with phosphotungstic acid solution or some other gelatin precipitant, it became a plain collodion membrane once more.

A matter which needs to be emphasized again is that diffusion through a protein-containing membrane is a very different phenomenon from diffusion through a plain membrane. Without the protein, solutions of multivalent cations give no measurable diffusion. Multivalent anions do; but the rate is enormously increased when the protein is present. This is indicated by the two curves shown in Fig. 1, one for an untreated membrane, the other for a treated one. It will be seen that in the latter case the diffusion is for each concentration of ferrocyanide about seven times as rapid as in the former. That this is not a difference in the membranes themselves was shown by comparing the same membrane before and after coating. Its reality also is shown by averages given in Table I.

The primary rôle of a membrane in electrostatic diffusion is to concentrate at its pores the charges borne by diffusible ions. It is useful to picture this concentration of electrical charges as due to

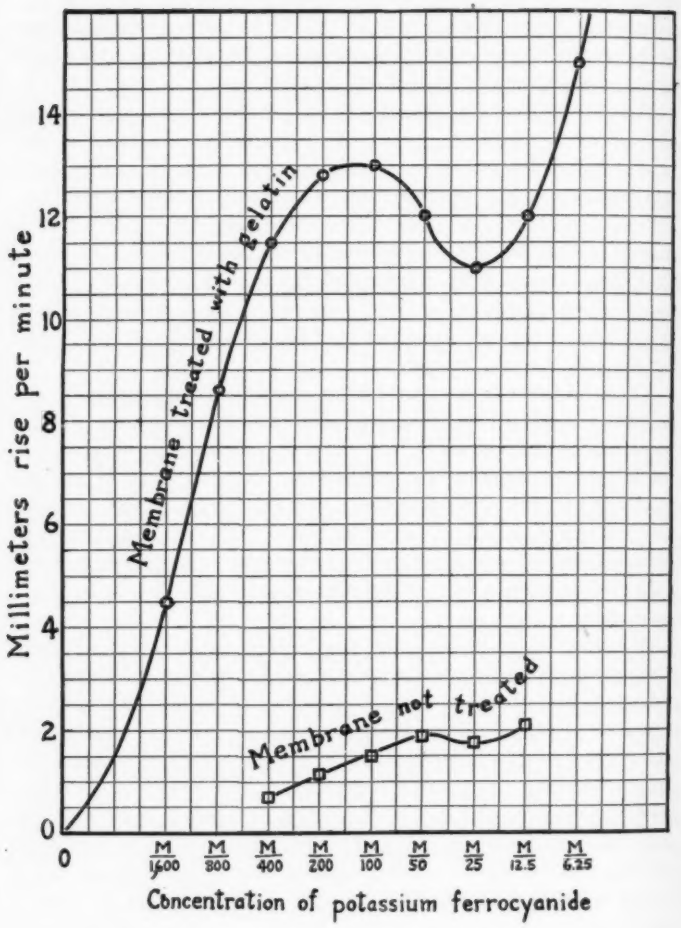


FIG. 1. Initial rates of diffusion of water through collodion membranes from pure water into various concentrations of potassium ferrocyanide. These curves illustrate the influence of concentration and the influence of treating the membranes with gelatin.

the membrane's properties as an imperfect ion sieve in the sense of Ostwald (48). In other words, the ions are repelled from the pores by virtue of the electrical charges of the membrane water surface; the ions in turn attract the water particles through the pores. This conception of a membrane is in accord with current ideas with regard to osmotic phenomena (Tinker (42)) and with regard to the membrane equilibrium of Donnan. It is probable that, as was first suggested by Quincke (15), electrostatic charges are not only localized or concentrated by the immense surfaces of a pored membrane, but also oriented by them. For purposes of calculation, he distinguished two components to the forces of diffusion; one in the direction of measureable water migration, the other in a plane at right angles to this migration.

TABLE I.

*Comparison of the Diffusion Rates from Pure Water through Untreated and Treated Collodion Membranes.*

The average rates of diffusion are given as mm. rise of water level in the manometer per minute.

| Salt solution. | Untreated membranes. | Membranes treated with gelatin. |
|---|---|---|
| M/32 sodium sulfate..... | 2.0 (Average of 7.) | 3.4 (Average of 11.) |
| M/64 potassium ferrocyanide..... | 3.6 (Average of 4.) | 7.5 (Average of 5.) |

A membrane which contains soluble proteins, such as an animal or vegetable membrane, or one which is treated with proteins, adds to the localization of forces. Why soluble proteins cling to pored surfaces, such as collodion membranes, is not fully understood but is probably also a result of the surface electrostatic charges. A protein changes the qualitative character of the membrane by virtue of being an electrolyte. No measureable potential difference is set up between such a membrane and pure water; but in the presence of a more highly ionized salt, the membrane has a great influence. This influence we may picture as due to an unequal distribution of the diffusible ions (Donnan equilibrium) in the vicinity of the protein particles. This unequal distribution of ions augments the local potential difference, because of the fact that the protein is a non-diffusible electrolyte. It is interesting to note that greater influences upon membranes

containing gelatin are exerted by cations than by anions. In other words, these membranes are now predominantly acid in character. It must be emphasized that the protein acts primarily by virtue of its ions, and not by virtue of its mechanical properties such as might change the pore size.

There is no reason for regarding the proteins as peculiar in their influence upon the rate of diffusion of water. They combine, however, two special properties; the ability to locate themselves permanently on a membrane, while they behave as though in true solution, and the ability to reverse their ionic charges relative to water at definite hydrogen ion concentrations. The latter quality is particularly interesting because of the fact that not only the sign of charge, but also the amount of charge and consequently the rate of diffusion, can be progressively increased by the addition of further acid or alkali, up to the limit of the acid- or base-binding capacity of the particular protein.

In evaluating the physiological significance of electrostatic diffusion, it is evident that only that which occurs in the presence of protein-treated membranes need be considered, for proteins are universal in living material. Moreover, they are the means of producing the most intensive diffusions known, and will therefore tell us what is the greatest rôle that diffusions of this sort may possibly play.

*2. Influence of Solutes.*—Having established the qualitative similarity in behavior of all membranes treated with a given protein, the influence of dissolved substances upon the diffusion of water was analyzed. No variation in diffusion rate other than that expected from the osmotic pressure was found with any non-electrolyte. With electrolyte solutions present, however, protein-treated membranes were influenced as regards the diffusion of water through them, by either cations or anions. These influences were of several kinds, the most important one being the sign of charge of the ions of greatest valence.

(*a*) Loeb (44) has presented convincing proof that in the presence of univalent and bivalent ions, the sign of charge of a protein-containing membrane is often reversed at the hydrogen ion concentration of the protein's least solubility. It is well known that this point varies not only for different proteins, but also for other different ions present, so that it is misleading to

regard this as a fixed "isoelectric" point. In our experiments gelatin and beef hemoglobin have been used, and in both cases the influence of salts upon the diffusion of water was nil at some point which was largely determined by the protein's point of least solubility. At approximately this hydrogen ion concentration there is a reversal of ionic charge, and, as Loeb (44) has shown in his diffusion experiments, cations are the effective ions on the acid side of the reversal point of the protein-containing membrane, anions on the alkaline side. This was confirmed in the present experiments for all the salts studied.

Triunivalent salts form an apparent exception to the principle of reversal of sign of charge at some such isoelectric point. Ferric chloride produced the same high initial rate of diffusion through treated membranes over a wide neutral range, and due to its strongly acid dissociation, alkali did not inhibit the effect except in amounts sufficient to precipitate the ferric ions. This effect of trivalent cations has been explained by Loeb (49) as due to the overwhelming of the negative charge which accompanies alkalinity by the strong positive charge of cation trivalency. An alternative statement of the case is that the proteinates of trivalent cations are not anions but cations in the neutral range. In other words, the point in the acid-alkali scale at which reversal of the protein's sign of charge occurs then differs considerably from the protein's point of least solubility.

The presence of free acid or free alkali in diffusing solutions has a relatively large influence. This influence is due only in part to the charge of the solute relative to the charge of the water which is in the pores of the membrane; it is due also to the change of ionic properties *of the protein* as the acid or basic groups respectively are neutralized by ions of opposite sign. The specific influence of acids and alkalies is manifested only when neutral electrolytes are also present. In other words, not only does the sign of charge change, but the valence effect increases as the valence of the protein increases, in both the acid and the alkaline direction; changes which are parallel to the protein's titration curve.

(*b*) The influence of electrolytes upon the rate of diffusion of water through treated membranes is most obviously correlated with the valence of the effective ions. As noted above, the pre-

dominantly effective ions are those whose ionic charge is opposite to that of the protein contained in the membrane. The multivalent ions which have been studied in these experiments are $Ca^{++}$, $Fe^{+++}$, $SO_4^{=}$, $HPO_4^{=}$, ferricyanide$^{\equiv}$, and ferrocyanide$^{\equiv}$.

It is instructive to compare the initial rates of diffusion for isotonic solutions of cations of different valences, all in isohydric solutions (Table II). It is readily noticed that univalent ions produce little or no diffusion, bivalent ions produce a considerable rate of diffusion, and that trivalent ions produce a much higher rate of diffusion. To find the numerical relationships between comparable initial rates of diffusion not only the hydrogen ion

TABLE II.

*Comparison of Rates of Passage of Water from Pure Solvent into the Given Solutions at Three Hydrogen Ion Concentrations.*

The solutions were contained in 50 cc. gelatin-treated collodion sacs. The rates of passage are indicated as mm. rise of water level in the manometer per minute.

| Salt solution. | Solution in M/2,400 HCl (pH 4). | Solution in distilled water (pH 6). | Solution in M/1,600 NaOH (pH 10) |
|---|---|---|---|
| M/32 NaCl | 2.0 | 0.0 | 0.7 |
| M/32 KCl | 1.1 | 1.4 | 2.3 |
| M/32 $KNO_3$ | 0.1 | 0.9 | 3.6 |
| M/72 $CaCl_2$ | 3.0 | 0.0 | 0.0 |
| M/50 $FeCl_3$ | 23.0 | 22.5 | 22.0 |
| M/32 sodium phosphate | 0.1 | 6.0 | 7.0 |
| M/32 $Na_2SO_4$ | 0.0 | 3.6 | 5.6 |

concentrations must be equalized, but also the anion concentrations. This is impossible to do, and approximate comparison merely allows us to hazard the guess that Hardy's (50) rule for the comparative influence of ionic valency upon protein solubilities may apply here, as it does to the solubilities of inorganic salts (Brönsted (51)). According to this rule the influences of isosmotic concentrations of various ions are proportional to the power represented by their valence numbers. But solubility is an equilibrated condition, while diffusion is often due to electrostatic forces which may lead to temporary diffusion away from chemical equilibrium.

(*c*) For the purpose of studying those individual properties of ions which affect the diffusion of water, varying amounts of acids

and alkalies were added to the solutions of several salts. In this way "titration curves" were obtained. None is here reproduced, since Loeb (44) has given many of them. It is apparent from those cases which we have studied that the same small concentration of an acid or an alkali influences diffusion to various extents, depending upon the particular salt present. Thus, among the univalent salts listed in Table II, in the acid range NaCl produces rapid diffusion, while $KNO_3$ exerts a larger influence in the alkaline range. KCl is intermediate between NaCl and $KNO_3$ in both ranges, indicating that both the particular anion and the particular cation play a part in every case. The titration curves turn out to be equally important with the concentration curves; from the latter alone, however, Loeb (52) showed that ions of a single family in the periodic table form series in the order of their atomic weights.

Arrhenius (53) and Nernst (39, 54) have related the influence of individual ions in diffusion to their mobility and to their activity in conducting electricity. These quantities, originally empirical corrections in measurements of ionic transport and electrical conductivity, are now usually correlated with the ionic radius and with the atomic number. The latter is therefore the basic relation to which these several individual ionic properties must be referred.

In the electrostatic diffusion of water, the effective concentrations of dissolved electrolytes are those which bathe the proteins in the membranes. But as the water actually passes, it evidently tends to carry certain solutes away from the membrane. It is obvious that the rate at which the solutes diffuse back toward the membrane may be a factor in determining the flow of water. From the studies of Arrhenius (53) we know that each species of ion in solution has a different rate of diffusion, and the membrane undoubtedly also influences their diffusion, so that the net influence must be a resultant of many effects, particularly where more than one solute is present. To measure the rate at which an electrolyte passes in the reverse direction to the water, as Loeb (34) did, probably measures only part of the desired quantity. Particularly in cases where the diffusion of water into the salt solution occurs rapidly, the concentration of salt at the membrane will be automatically decreased. There is therefore a maximum rate of passage, which depends upon the velocity of diffusion of

the salt against the water current back to the membrane. For this reason it is possible that the effect of trivalent and quadrivalent ions is less than the true influence of the given concentration of them. The influence of the diffusion rate of the salt upon the diffusion rate of the water would have to be calculated in any complete account of diffusion forces.

It must be clearly understood that the electrostatic forces are eventually neutralized under ordinary conditions when all diffusions have proceeded to their limits. Equilibrium is therefore not changed, and electrostatic diffusion gives no indication with regard to it. Unless there is some mechanism for maintaining a potential difference between two localities, a flow of materials will not continue.

## V.

## *Diffusion between Mixed Electrolyte Solutions through Membranes Treated with Gelatin.*

### *1. Between Two Solutions of the Same Electrolyte.*

So far we have considered the velocities of electrostatic transport of water through protein-treated membranes between pure water and simple electrolyte solutions. The next step was to correlate with these velocities the transport of water between two solutions of the same simple electrolytes.

Since the protein contained in a treated membrane is not polarized, and is in the pores of the membrane, no net diffusion of water is to be expected when identical solutions are on either side. This was found to be the case. The membrane *per se* has therefore no influence upon diffusion; it acts only when an electrostatic difference, *i.e.* a diffusion potential, exists between the liquids on its two sides.

For the case of solutions of the same solute in different concentrations, a competition between the two solutions is set up. As shown in Fig. 2, there results a diminution of the intensity of diffusion. In these data we have the interrelationship of the competing electrostatic forces. Can this interrelationship be expressed simply? It is evident from Fig. 2 that the diffusion rates between the competing concentrations are not directly

proportional to the concentrations. This is to be expected, since the diffusion rates from pure water are not directly proportional to concentrations (Fig. 1). In attempting to find a relationship we have proceeded in two ways.

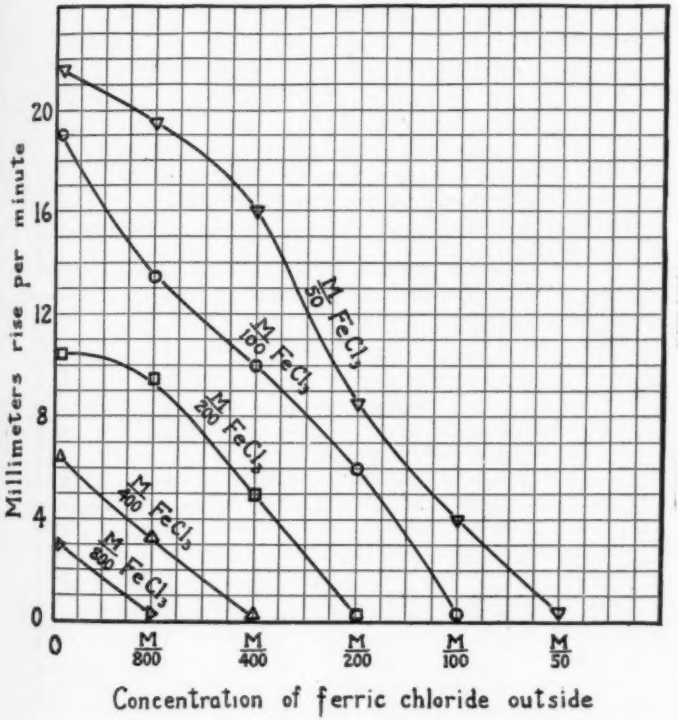


FIG. 2. Initial rates of diffusion of water between pairs of solutions of ferric chloride. Each curve connects all the points where like concentrations were on one side of the gelatin-treated collodion membrane, the abscissæ represent concentrations on the other side of the membrane.

One empirical method of correlation is to subtract the rate for pure water *versus* outside solution from the rate for pure water *versus* inside solution. The difference often approximates the rates actually determined for the passage of water from outside

solution to inside solution. This agreement over a considerable range of concentrations is shown in Table III for the two salts ferric chloride (same data are represented in Fig. 2) and sodium sulfate.

TABLE III.

*Comparison of Observed Diffusion Rates between Solutions of Different Concentrations of the Same Salt, with Values Calculated by (a) Subtracting the Rate for the Passage of Pure Water into the Lower Concentration from the Rate for the Passage of Pure Water into the Higher Concentration, and (b) Considering the Diffusion between the Two Concentrations as Equivalent to the Passage of Pure Water into a Concentration Equivalent to the Difference in the Two Concentrations.*

The rates of diffusion are indicated as mm. rise of water level in the manometer per minute.

| Salt. | Molar concentration. | | Observed. | Calculated *a*. | Calculated *b*. |
|---|---|---|---|---|---|
| $FeCl_3$ | 0.02 | *vs.* 0.01 | 4.0 | 2.5 | 19.0 |
| | 0.02 | 0.005 | 8.5 | 11.0 | 20.5 |
| | 0.02 | 0.0025 | 16.0 | 15.0 | 21.0 |
| | 0.02 | 0.00125 | 19.5 | 18.5 | 21.2 |
| | 0.01 | 0.005 | 6.0 | 8.5 | 10.5 |
| | 0.01 | 0.0025 | 10.0 | 12.5 | 15.0 |
| | 0.01 | 0.00125 | 13.5 | 16.0 | 17.2 |
| | 0.005 | 0.0025 | 5.0 | 4.0 | 6.5 |
| | 0.005 | 0 00125 | 9.5 | 7.5 | 8.6 |
| | 0.0025 | 0.00125 | 3.3 | 3.5 | 3.0 |
| $Na_2SO_4$ | 0.04 | *vs.* 0.02 | 1.0 | 0.5 | 2.0 |
| | 0.04 | 0.01 | 1.7 | 1.0 | 2.3 |
| | 0.02 | 0.01 | 0.5 | 0.5 | 1.5 |
| | 0.01 | 0.0025 | 1.2 | 1.2 | 1.4 |
| | 0.005 | 0.0025 | 0.8 | 1.1 | 0.3 |
| | 0.0025 | 0.00125 | 0.1 | 0.1 | 0.2 |

The second relationship is obtained by regarding *e.g.* diffusion from M/200 into M/100 as equivalent to diffusion from pure water into M/200 (Table III). This method is equally unsatisfactory, and no exact relationship is apparent.

As will be shown below, however, we are dealing in diffusion with electrostatic forces and not with chemical masses, so that concentration is in reality a secondary correlative. This is illustrated by the fact that, while Fig. 1 shows that M/100 potassium ferro-

cyanide attracts water more rapidly than M/12.5, yet in actual experiment the latter draws water from the former at a rate almost as rapid as from pure water.

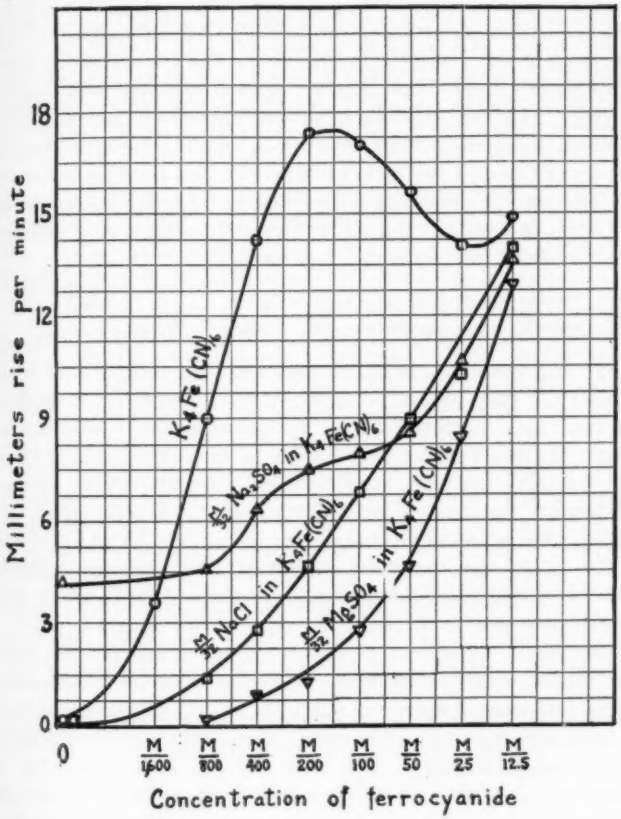


FIG. 3. Initial rates of diffusion through a gelatin-treated collodion membrane from pure water into various concentrations of potassium ferrocyanide, without and with another salt in the same solution.

*2. Between a Solution of Two Electrolytes and Pure Water.*

If we compare the diffusion of pure water into a pure multivalent solution such as potassium ferrocyanide with that into a mixture of ferrocyanide with sodium chloride, we get two concentration curves such as are shown in Fig. 3. The presence of the uniunivalent salt NaCl tends to decrease the attraction for water at the protein-treated membrane. At first sight there appears to be little quantitative relation between these two curves, but by empirical comparison the relation was found to be quite simple. The square root of the ratio of twice the NaCl concentration to the ferrocyanide concentration proved to be equal at every concentration of ferrocyanide to the ratio of the ordinates of diffusion rate read from the two curves. That is

$$\frac{\text{Diffusion rate into pure ferrocyanide}}{\text{Diffusion rate into ferrocyanide + NaCl}} = \sqrt{\frac{4\,[\text{NaCl}]}{2\,[\text{K}_4\,\text{Fe(CN)}_6]}}$$

The agreement of calculated and observed results is shown in Table IV. There can be no doubt that the diffusion potentials in the two cases are functions of the concentrations. Similar procedure for the other curves shows other simple relationships. For the bibivalent salt $MgSO_4$ the ratio of diffusion rates is proportional to the concentrations, while for the unibivalent salt $Na_2SO_4$ the ratio is the same as for NaCl (Fig. 3 and Table IV). In the latter case, however, we correct the observed diffusion rate by subtracting the diffusion rate for $Na_2SO_4$ alone; also remembering that each molecule contains two cations.

Now these numerical relationships were arrived at purely empirically. But it is evident that we have to do with something which conforms to a general rule. It is the relative proportions of both the cations and the anions in the solution which counts in producing diffusion. The proof of such a deduction lies in applying it to other data. When this was done we found that the relationship for NaCl and ferrocyanide holds for concentrations of both salts between M/32 and M/512 (data not given in detail) providing the molar concentrations of ferrocyanide do not exceed those of NaCl. In the latter case the effect of NaCl approaches zero, thus departing from the formula. In other words, the NaCl never acts to increase the rate of diffusion over that for ferro-

cyanide alone. Outside of the above concentration limits, it is not feasible to make comparisons, for in more concentrated solutions the osmotic pressure influence is large, and in more dilute solutions the diffusion rates are too small for accurate comparison. Application of the formula to another salt than ferrocyanide is shown in Fig. 4. Here the rates for the diffusion of pure water have been compared, in the first two curves, for pure ferricyanide with those for ferricyanide plus M/32 NaCl. We find (Table V) that the calculated ratios agree with the observed ones merely by

TABLE IV.

*Comparison of Diffusion Rates into Pure Ferrocyanide Solutions with Rates into Mixed Solutions.*

The ratios of the rates as read from Fig. 3 are compared with ratios calculated by the empirical formulas indicated below.

| Concentration of ferrocyanide. | Ratio of rate into ferrocyanide to rate into ferrocyanide plus M/32 NaCl. | | Ratio of rate into ferrocyanide to rate into ferrocyanide plus M/32 $Na_2SO_4$. | | Ratio of rate into ferrocyanide to rate into ferrocyanide plus M/32 $MgSO_4$. | |
|---|---|---|---|---|---|---|
| | Observed. | Calculated. | Observed. | Calculated. | Observed. | Calculated. |
| M/16 | 1.09 | 1.00 | 1.77 | 1.41 | 1.30 | 1.00 |
| M/32 | 1.40 | 1.41 | 2.62 | 2.00 | 2.12 | 2.00 |
| M/64 | 1.99 | 2.00 | 4.13 | 2.83 | 4.02 | 4.00 |
| M/128 | 2.90 | 2.83 | 4.97 | 4.00 | 7.90 | 8.00 |
| M/256 | 4.2 | 4.0 | 5.7 | 5.6 | 13.8 | 16.0 |
| M/512 | 5.5 | 5.6 | 8.1 | 8.0 | 30.4 | 32.0 |
| M/1,024 | 6.8 | 8.0 | 11.3 | 11.3 | | |
| Formula for calculated ratio..... | $\sqrt{\frac{4[NaCl]}{2[K_4Fe(CN)_6]}}$ | | $\sqrt{\frac{8[Na_2SO_4]}{2[K_4Fe(CN)_6]}}$ | | $\frac{4[MgSO_4]}{2[K_4Fe(CN)_6]}$ | |

substituting the coefficient 3 in place of 4 in the ferrocyanide formula. From this it becomes probable that the coefficients in the numerator correspond to the valence of the predominant ion, and the coefficients in the denominator correspond to the valence of the "depressing" ion of opposite sign. The general formula for anion diffusion then is:

$$\frac{\text{Diffusion rate into pure salt}}{\text{Diffusion rate into mixed salt}} = \left(\frac{\text{Valence of effective anion} \times \text{concentration of depressing cation}}{2 \times \text{concentration of effective anion}}\right)^{\frac{\text{valence of depressing cation}}{2}}$$

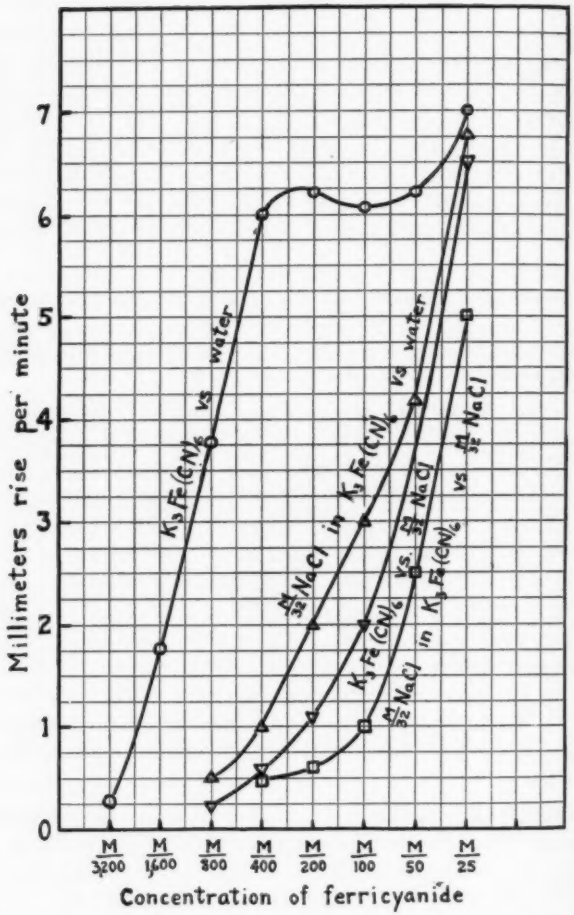


FIG. 4. Initial rates of diffusion of water through a gelatin-treated collodion membrane into various concentrations of potassium ferricyanide. Sodium chloride was added either to the ferricyanide or to the water or to both.

A numerical relationship of this sort leads one at first to consider it an instance of the "mass law." But the general relationship obtained does not on analysis correspond to the mass law, even introducing all modern modifications of it. An interpretation of it we have developed from a suggestion of Dr. W. A. Patrick. He suggested to us that in the pores of a membrane the maintenance of electrostatic potentials between the water and the membrane depend upon the non-conductance of the liquid medium. Let us picture the case of a ferrocyanide solution where the membrane is negatively charged and the water in the pores is positively charged. Our supposition is that anything

TABLE V.

*Comparison of Diffusion Rates from Pure Water into Pure Ferricyanide Solutions with Rates into Similar Solutions Containing M/32 NaCl.*

The data are read from Fig. 4. The ratio of rates is compared with that calculated from the formula $\sqrt{\frac{3[NaCl]}{2[K_3Fe(CN)_6]}}$.

| Concentration of ferricyanide. | Diffusion rate into pure ferricyanide. | Diffusion rate into ferricyanide plus M/32 NaCl. | Ratio of observed diffusion rates. | Calculated ratio of diffusion rates. |
|---|---|---|---|---|
| | *mm. per min.* | *mm. per min.* | | |
| M/32 | 6.6 | 5.8 | 1.15 | 1.22 |
| M/64 | 6.2 | 3.6 | 1.72 | 1.73 |
| M/128 | 6.2 | 2.6 | 2.38 | 2.45 |
| M/256 | 6.1 | 1.6 | 3.8 | 3.5 |
| M/512 | 5.0 | 0.8 | 6.2 | 4.9 |
| M/1,024 | 2.0 | 0.4 | 7.2 | 6.9 |

which reduces the electrical resistance of the water will reduce the electrostatic tension which causes the flow of water. Such reduction actually is caused by the presence of conducting particles in the water, as Quincke (15) and Coehn and Franken (55) have shown.

The relationship between the conductance of the solution and the rate of water transport by an electric current is expressed in an equation of von Smoluchowski (56). His expression shows that the rate of liquid transport is directly proportional to (*a*) the dielectric constant of the solution in the pores, (*b*) the specific resistance of the solution in the pores, (*c*) the electrical current traversing the membrane, and (*d*) the electrostatic potential

difference between the *pore walls* and the solution in the pores. This expression is an empirical statement merely of the fact that reduction in any one of these four properties reduces the electrical transport of water. Upon our view the property in question is most accurately expressed as specific resistance of the solution in the pores.

An inspection of our data (Fig. 3) indicates, however, that the reduction in specific resistance is due chiefly to the positively charged solute particles. This seems to be a corollary of the fact that the water is positively charged. Thus, the quantitative relationship to cation concentrations is confirmed by the fact that bivalent cations ($Mg^{++}$) have exponential value in reducing the electrostatic potential conferred by trivalent and quadrivalent anions.

This view of what happens in membrane pores also enables us to understand the concentration curves such as those given in Fig. 1. The reduction in diffusion potential which occurs in concentrations greater than M/100 was affirmed by Loeb to be due to the cations; and this we believe. But, on the basis of the mass law, it is impossible to account for the effect quantitatively, for by "Keplerizing" to find quantitative interrelationships between the concentrations of cations and the concentrations of anions, nothing resembling a mass equation was found possible. But the reduction of diffusion potential by electrically conducting cations is what must happen whenever the cation concentration becomes great enough to reduce the electrical resistance of the water. The least effective concentration of cations would seem to bear a specific relationship to the pore size; the ions must be distributed with sufficient frequency, or else in sufficiently large valences, to average some constant number or intensity per unit of pore length before the electrical current which transports the water is effectively reduced. This is confirmed by the fact that, if we compare for various salts the cation concentrations which just overcome the anion diffusion, we find that all such solutions conduct approximately proportional amounts of positive electricity. This comparison is made by selecting for correlation those concentrations of various salts which give the maximum electrostatic diffusion rate. This also accounts for the fact that salts such as $MgSO_4$ do not cause electrostatic diffusion, while heterovalent salts of the type $Na_2SO_4$ do.

We believe, then, that the "depression" due to the ion of opposite charge to that which causes the predominant diffusion is actually due to the reduction in the dielectricity of the water. In electrostatic diffusion there is never "antagonism" between two ions of the same sign, so far as we have discovered; but there is always antagonism between cations and anions. Since we cannot have one present without the other, reduction of diffusion potentials occurs after any addition of salts.

*3. Between Two Solutions of Different Electrolytes.*

What will be the result if the depressing salt (*e.g.* sodium chloride) is, in one solution (outside), separated by the protein-treated membrane from the predominant salt (*e.g.* ferricyanide) (inside)? The results of one set of such experiments are indicated in Fig. 4. It is evident that in this case the NaCl has a much greater retarding effect than when in the same solution with the ferricyanide. If it acted merely by virtue of its relative mass which bathes the protein, then its effect would be smaller than before. But its effect is larger, and this can be pictured as due to an electrostatic force which tends in the opposite direction. It is apparent from this that NaCl solution opposed to a solution of a multivalent salt has an attracting power for water, and this is illustrated further in Fig. 5; whereas NaCl solutions less concentrated than M/16 are almost unable to attract pure water through collodion membranes. Only when opposed to weak concentrations of such multivalent solutions as ferric chloride (which are, also, slightly acid) is water rapidly drawn into them. There is no theory to account for this, although the relative charges then conferred on the membrane can easily be pictured so as to conform with the electrostatic conception.

To formulate the influence of two opposing solutions we have not been able to obtain a quantitative expression; apparently both geometrical and arithmetic functions (ratios and differences) are involved. But it is certain that the influence of the ions of greater valence is always reduced by a larger amount when the oppositely charged ions of lower valence are in opposing solutions than when they are in the same solution.

The diffusion of water between a solution of a non-electrolyte and that of an electrolyte is readily understood. Here we can

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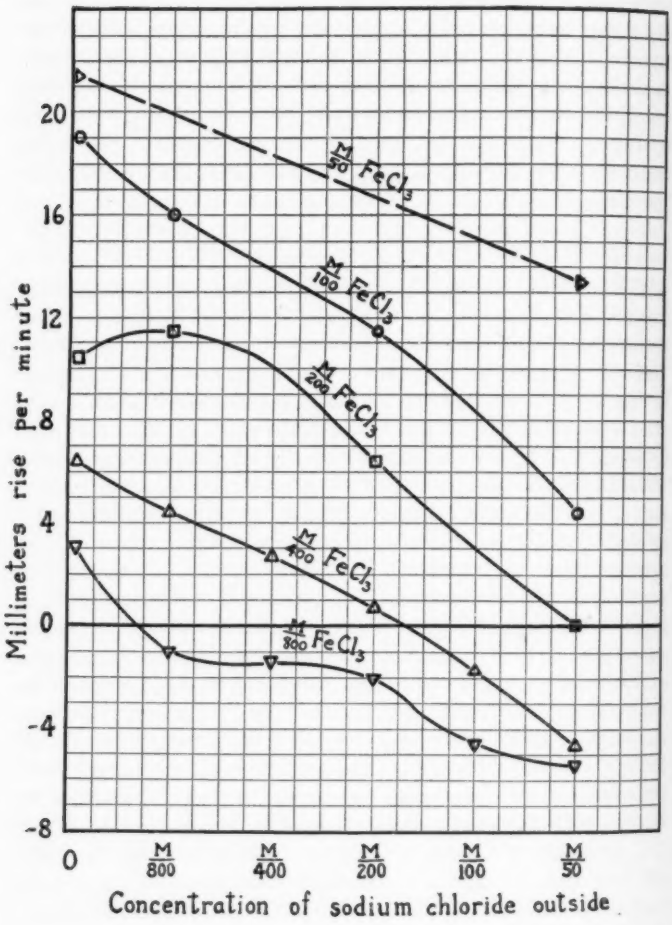


FIG. 5. Initial rates of diffusion of water between solutions of ferric chloride and solutions of sodium chloride. Each curve connects all the points where like concentrations of the ferric salt were on the inside of the gelatin-treated collodion membrane.

predict the resulting rate by taking the difference of the rates for the migration of pure water into each of the solutions. In this way one can furnish striking demonstrations of the electrostatic effect; thus a M/400 ferric chloride solution will draw water away from a M/1 sucrose solution, though its osmotic pressure is only one two-hundredth as great.

The above analysis of the effect of mixed electrolytes upon the diffusion of water must be the basis for understanding the rôle of solutes and the rôle of the membrane in all electrostatic diffusion. Of more practical importance is the result that small concentrations of multivalent ions such as are found in physiological fluids and in living substance are shown to be incapable of bringing about conspicuous electrostatic diffusions, because of the much higher concentrations of univalent ions present with them. Moreover, the differences in hydrogen or hydroxyl ion concentration among living units are so limited that they cannot lead to any great amount of electrostatic diffusion.

A number of experiments were performed in which various combinations of $CaCl_2$, NaCl, KCl, and $NaHCO_3$ were opposed to each other, all giving immeasureably slow diffusions of water between them. The chief error in inferring the situations in living systems from those in the ones studied, is due to the fact that living membranes are in general less permeable to dissolved electrolytes than are collodion membranes. This will be taken up in another paper. From the present experiments it seems unbelievable that ions alone, *i.e.* by virtue of their differential distribution, are of themselves able to lead to electrostatic diffusions of water in living tissues; and the analogies of Oker-Blom (57), Hamburger (29), Girard (58), Bethe (59), and others are only analogies, because the phenomena to be explained have to do with fluids which lie within physiological limits of composition. Other means of current production are certainly present in living tissues, if water is there distributed by electrostatic agencies.

I am indebted to the Elizabeth Thompson Science Fund for a grant from which some of the expenses of this investigation were defrayed. To Mr. G. D. Newton and to the University of Pittsburgh I am indebted for technical assistance in some of the earlier experiments.

VI.

## SUMMARY.

1. The diffusion of water into solutions of electrolytes is relatively infrequent and slow through membranes other than those containing non-diffusible electrolytes such as proteins.

2. The diffusion of water through gelatin-treated collodion membranes is correlated with the electrical properties of each ion in the portions of the solution which bathe the membrane. This is particularly evident in mixed solutions, where the rates of diffusion can be calculated from the ratios of the concentrations of the effective ions which are present.

3. The predominant influence of multivalent ions in leading to diffusion is rendered very small whenever larger concentrations of ions of opposite sign of charge are present. This is believed to be due to the increased electrical conductivity of the diffusing solution.

4. The diffusion of water between two concentrations of the same salt and between two different salts was studied. The diminution of the influence of predominant ions was in this latter case still greater than when the salts were combined in the same solution.

5. Between solutions which differ very considerably, but within the range of composition of those fluids found within living organisms, no measureable diffusion through gelatin-treated collodion membranes occurs. Such ions alone do not, therefore, furnish potentials and currents such as are necessary to accomplish irreciprocal water transport in living tissues.

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# NATURE OF HEAT DENATURATION OF PROTEINS.

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Chick and Martin (1) have shown that the heat coagulation of proteins consists of two distinct processes; *viz.*, (*a*) denaturation, the alteration of the protein under the influence of heat, and (*b*) agglutination, the separation of the altered protein in a particulate form. Agglutination, in which hydrogen ion concentration and electrolyte contents play important rôles (2), is now a fairly well understood phenomenon of colloidal chemistry, while the significance of denaturation is still obscure.

Although denaturation is made evident by agglutination, the latter is not a necessary consequence of the former. In this paper we are concerned only with the alteration which heat-coagulable proteins undergo under the influence of heat, and we will designate it as heat denaturation to distinguish it on the one hand from denaturation by other agencies and on the other from heat coagulation, which would include the physical process of agglutination. No little confusion in the literature has resulted from the failure to distinguish the two processes comprised in heat coagulation of proteins.

The word denaturation is used loosely to designate the change of proteins from a soluble to an insoluble form brought about by a large variety of chemical and physical agents, including acids, alkalies, alcohol, acetone, salts of heavy metals, alkaloidal reagents and dyes (3), and heat, light, and pressure (4). Chick and Martin (5) consider heat denaturation as a reaction between protein and water which implies in all probability a hydrolysis. Robertson,[1]

[1] Robertson (4), pp. 304–311.

on the contrary, supports the old view of Hofmeister that heat denaturation is a phenomenon of dehydration. Excluding the possibility of intramolecular rearrangement for which there is no experimental evidence, the heat denaturation must then be either a hydrolysis or a dehydration.

In a previous paper (6) we have shown that when a protein is denatured by dilute acids and alkalies at ordinary temperatures, an increase occurs in the acid- and base-binding powers as well as in the reactivity toward the phenol reagent of Folin and Denis. Since both these changes are known to accompany the hydrolysis of proteins, we concluded that the denaturation of proteins by dilute acids and alkalies is a hydrolytic process. We have reason to believe that the denaturation of proteins in the presence of $H_2O$ and under the influence of most agents is a hydrolytic process. In this paper we will show that the heat denaturation of proteins is exactly similar to the denaturation of proteins by acids and alkalies and that the two apparently different processes are one, that of hydrolysis. Before presenting our experiments, however, we will review briefly the evidences bearing upon the dehydration theory.

Robertson[2] has found that casein dissolved in anhydrous formic acid is not precipitated by cupric chloride until sufficient water has been added to cause a change of the color of the solution from green to blue. If alcohol is added instead of water, no precipitate is formed until the color of the solution has changed from green to brown. Since water and alcohol are obviously hydrating and dehydrating agents, respectively, and since the changes of the color of cupric chloride indicate changes in the degree of hydration as generally believed, Robertson concluded "that protein may be thrown out of solution by electrolytes in two grades of hydration, the one of high, and the other of very low hydration." (The former process he called precipitation, and the latter, coagulation). From these experiments he concluded also that the process of heat coagulation is not one of hydration but of dehydration of the protein.

Robertson's interpretation of the precipitation of casein by cupric chloride is probably correct, but there is little similarity between the heat denaturation of proteins in an aqueous solution and the precipitation of casein in anhydrous formic acid solution with cupric chloride to warrant the transfer of the conclusion from one process to the other. The precipitate in Robertson's experiments is cupric caseinate, while the heat-coagulated protein is protein as such. The precipitation of casein from formic acid solution by cupric chloride and water or alcohol is a revers-

[2] Robertson (4), pp. 125–129.

ible process, for the precipitate produced by water redissolves on adding more formic acid, and that produced by alcohol redissolves on adding water. Heat coagulation is, on the contrary, an irreversible process.

Robertson argues that the irreversibility of heat coagulation (denaturation) is only apparent, and he cites the observation of Corin and Ansiaux (7) that if a solution of protein be cooled and vigorously shaken just as the first traces of heat coagulation (flocculation) appear, the incipient coagula will pass again into solution.

We have repeated this experiment with only negative results, but it has occurred to us that if the denatured protein will revert to the original soluble form by cooling and shaking after it has flocculated to give a turbid solution, it should revert so much more readily, if the denatured protein is not allowed to flocculate at all. We have heated a solution of egg albumin to which a minimal amount of acid or alkali (0.5 cc. of 0.1 N HCl or NaOH for 50 cc. of 0.1 per cent egg albumin solution) has been added to prevent flocculation.[3] On neutralizing a part of the heated solution the protein was precipitated, showing that denaturation has occurred. The remainder of the solution was cooled and left in an ice box under sterile conditions. After 2 months the solution was neutralized and the protein precipitated as before. The heat-denatured protein, therefore, cannot revert to its original form and the process is accordingly an irreversible one.

As another evidence of the reversible character of heat denaturation Robertson cites the observation of Berczeller (8) that the surface tension of a protein solution which is so salt-free as not to coagulate on heating, nevertheless diminishes on heating, and rises again on cooling and standing. The true explanation of this is found in Berczeller's paper. Although his protein solutions did not flocculate, they did become turbid, and the rise of the surface tension took place as the result of the protein particles becoming large enough to separate out from the solution. This is,

[3] It cannot be urged that the denaturation of the protein which occurs in solutions containing such a minute amount of acid or alkali is essentially different from that which occurs in a neutral solution or at the isoelectric point of the protein. Since proteins are denatured in acid as well as in alkaline solutions, it follows that the rate of denaturation must pass through a minimum as we go from the acid to the alkaline side. There is no reason to believe, however, that the character of the reaction should change abruptly at any point in the pH range. Furthermore, heat coagulation as ordinarily carried out in practice certainly does not represent a reaction of the isoelectric protein.

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in fact, a proof of the irreversibility rather than of the reversibility of heat denaturation of proteins.

Robertson (9) found that the solubility of casein in alkaline solutions was considerably augmented by carrying out the process of solution at temperatures above 40°C. Osborne (10) observed an increase in alkalinity and electrical conductivity of caseinate solutions upon heating. In explaining these facts Robertson assumes that there is an equilibrium between protein and water of the type

$$HXOH + HXOH \rightleftharpoons HXXOH + H_2O$$

and that the influence of heat on protein consists in shifting the equilibrium from left to right, that is, in the direction of higher complexes, with consequent decrease in the base-binding power and increase in the amount of casein dissolved for a given amount of alkali. This explanation is plausible, but the fact that in none of the solutions in which more casein was dissolved at higher temperature than would be dissolved at room temperature did he observe any appreciable tendency towards precipitation of casein on cooling, seems to show that the casein has undergone some decomposition. Even granting that his explanation is correct as applied to casein, the conclusion is still unwarranted that heat denaturation is the result of repeated condensation of the protein molecule, since free casein itself is insoluble (10) and is like a denatured protein.

Chick and Martin (1) have shown that heat coagulation (denaturation) is a monomolecular reaction. This would exclude the possibility of polymerization which is a reaction of higher order.

That water is essential for the heat denaturation of proteins has been known for some time. According to Wichmann (11) albumin crystals can be heated to 150° in the dry condition without change. Osborne (12) found in the case of proteins soluble in alcohol that such alcoholic solutions may be boiled without precipitating the protein, but, if diluted with water, some coagulation occurs when the solution is heated to the same temperature. Confirmatory to this observation, Chick and Martin (5) found that crystallized egg albumin was completely soluble after 5 hours heating at 120°, although in the presence of steam the albumin became completely insoluble in a few moments. Robertson tried to harmonize this observation of Chick and Martin with his dehydration theory by assuming that dry protein has been deprived of the elements of water in its end $-NH_2$ and $-COOH$ groups, and that these, consequently, cannot react to form polymers of the protein. We have already pointed out that heat denaturation, being monomolecular, cannot be a process of polymerization. If heat denaturation is a process of dehydration at all, it can only be one by internal neutralization of the $NH_2$ and COOH groups. The protein should then be denatured as it loses water on drying which is contrary to fact.

Nor can the failure of the dry protein to be denatured be accounted for by the reaction occurring in a solid system which is extremely slow

as Robertson contends further. Chick and Martin found that egg albumin was completely denatured in the presence of steam. Here the albumin was not dissolved in water, and the reaction occurred not in a liquid system, but in a solid system, or between gas (water vapor) and solid according to our interpretation.

There is another line of evidence which Robertson mentioned in support of his theory. From the observations of some investigators that heat is evolved when a protein is hydrolyzed, Robertson concludes that the hydrolysis of protein is an exothermic reaction and that, therefore, according to van't Hoff's principle of mobile equilibrium, the effect of heat must be to shift the equilibrium point of the system protein-amino acids in the direction of condensation. Since the heat of reaction of protein hydrolysis is extremely small, the reliability of the few available data is open to question, especially as some other observers have failed to detect any evolution of heat. Even if the evolution of heat in protein hydrolysis is real, we still have to know the secondary reactions which occur and the amount of substances entering into these reactions, before the heat of the main reaction—hydrolysis—can be calculated. In the experiment on tryptic digestion of casein reported by Henderson and Ryder (13) for instance, some heat would be produced from the neutralization of sodium carbonate by the phosphoric acid and dicarboxylic amino acids liberated from casein, and the slight evolution of heat which they observed is certainly no convincing proof of the exothermic character of protein hydrolysis.

The fact that proteins are hydrolyzed far more readily at 100° than at ordinary temperature shows that heat favors hydrolysis. Whether this is due to the acceleration of the reaction toward equilibrium or to a shift of its equilibrium point in favor of hydrolysis is a matter of no moment in the present discussion.

It is seen from the above review that all the available evidence is in favor of the hydrolysis theory of heat denaturation of proteins.

We have observed many points of similarity between the denaturation of proteins by heat and that by dilute acids and alkalies at ordinary temperatures. The denatured protein in either case is insoluble in neutral water or salt solution or, more accurately, at the isoelectric point of the protein, but redissolves in the presence of slight excess of acid or alkali. The velocity of denaturation in both processes is increased by increasing the hydroxyl ion concentration in alkaline solution, and the hydrogen ion concentration in acid solution (1, 6, 14). In both processes a decrease of hydrogen ion concentration occurs in acid solution and a decrease of hydroxyl ion concentration in alkaline solution (1, 6, 14). We have found further that the chromogenic value of

proteins (when allowed to react with the phenol reagent of Folin and Denis) increases when denatured by heat as well as when denatured by acids and alkalies, and that the increase in the case of heat-denatured protein does not disappear on cooling, showing the irreversible character of the reaction. Non-protein substances are liberated when the protein is denatured by either process.

The similarity between heat denaturation and denaturation by acids and alkalies has been observed not only in the general features mentioned above, but also in certain anomalous cases. We have reported previously that horse serum albumin remains unchanged in 0.05 N HCl solution as far as solubility is concerned (also 1 per cent in $Na_2SO_4$, which, however, has no bearing upon the present discussion), even after the solution has stood 2 months, although in 0.02 N HCl the albumin is denatured and precipitates out, with or without neutralization of the acid. Exactly the same relation holds for the heat denaturation of horse serum albumin. The albumin is not denatured by heat in 0.05 N HCl solution, although in 0.02 N HCl it is denatured and the denatured protein flocculates with or without neutralization of the acid. Pigeon's egg albumin undergoes chemical change in 0.05 N NaOH solution as shown by a marked production of ammonia and a decrease of titrable alkalinity, but the solution gives no precipitate or turbidity on neutralization. The behavior of pigeon's egg albumin on heating is exactly the same. Neutral or acid solutions become turbid on heating, but when an alkaline solution is heated, no opalescence is observed even after neutralization. We cannot at present explain these anomalies, but whatever be the underlying reason, they go to show that the denaturation of proteins by heat and that by dilute acids and alkalies are the same kind of process.

Chick and Martin have shown that heat denaturation is not purely a temperature effect which begins suddenly at a particular temperature, the so called coagulation temperature, but that it is a chemical reaction with a large temperature coefficient. The reaction which underlies the heat denaturation of proteins, must, therefore, take place also at ordinary temperatures, though at an extremely slow rate. In the presence of sufficient hydrogen ion or hydroxyl ion concentration, however, the reaction is accelerated. Conversely, the denaturation of the protein in faintly acid or alkaline solution is very slow at ordinary temperatures, but the reaction is accelerated by a rise in temperature.

EXPERIMENTAL.

*Effect of Heat Denaturation on Chromogenic Value of Protein.*—Three tubes of hen's egg albumin solution were prepared as follows: (1) 1 cc. of 1 per cent albumin plus 15 cc. of $H_2O$; (2) 1 cc. of 1 per cent albumin plus 15 cc. of $H_2O$ plus 0.1 cc. of N HAc; and (3) 1 cc. of 1 per cent albumin plus 15 cc. of $H_2O$ plus 0.1 cc. of N $Na_2CO_3$.

Tubes 2 and 3 were placed in boiling water for 5 minutes. After cooling 0.5 cc. of phenol reagent and 3 cc. of 20 per cent $Na_2CO_3$ were added to all three tubes, made up to 25 cc., and mixed. Using Solution 1 as a standard set at 20 mm., Solutions 2 and 3 read 16.1 and 13.0 mm., respectively, in a Duboscq colorimeter. The HAc and $Na_2CO_3$ were used to prevent the flocculation of the denatured protein which would otherwise interfere with the colorimetric determination. Heating the solution for 10 minutes gave practically the same results, showing that the denaturation has long been completed. In another experiment the solutions were cooled, and the protein was precipitated with 2 cc. each of 10 per cent sodium tungstate and 2/3 N sulfuric acid, and the precipitates were redissolved in 2 cc. of the tungstate for the determination of the chromogenic value. Practically the same results were obtained. These experiments show (1) that heat denaturation increases the chromogenic value of the protein, (2) that the protein denatured in acid solution differs from that denatured in alkaline solution, and (3) that the increased chromogenicity lies mainly in the protein itself, although some non-protein chromogenic substances are liberated, as the following experiment shows.

*Liberation of Non-Protein Chromogenic Substances from Protein on Heating.*—20 cc. of 1 per cent hen's egg albumin solution plus 25 cc. of $H_2O$ in a 100 cc. Erlenmeyer flask were heated under cover for 10 minutes in boiling water. After cooling, 2.5 cc. of 2/3 N sulfuric acid were added, followed by 2.5 cc. of 10 per cent sodium tungstate. The resulting precipitate was filtered. A non-protein filtrate was prepared similarly from an unheated albumin solution. To 35 cc. of each filtrate were added 0.25 cc. of phenol reagent and 3 cc. of 20 per cent sodium carbonate solution. Compared with suitable standards the color intensity of the filtrate from the unheated albumin was found to correspond to 0.14 mg. of tyrosine while that from the heated albumin corresponded to 0.20 mg. of tyrosine. Calculation shows about 0.43 mg. of "tyrosine" was split off from 1 gm. of albumin.

*Increase in Acid- and Base-Binding Powers of Protein Attending Heat Denaturation.*—Chick and Martin have shown that a decrease of hydrogen ion concentration takes place when a protein is coagulated in acid solution, and a decrease of hydroxyl ion in alkaline solution. This can be simply demonstrated in the following way. Place in each of four test-tubes 5 cc. of 0.2 per cent egg albumin solution. Add to two tubes 0.1 cc. of 0.1 N HCl and 1 drop of methyl red. To the other two tubes add 0.1 cc. of 0.1 N NaOH and 1 drop of phenolphthalein. Place one tube of each pair in boiling water for a few minutes and cool. Compared with the unheated solutions the change in reaction of the heated solutions is striking.

*Anomalous Behavior of Horse Serum Albumin on Heating in Acid Solution.*—In the following set of experiments it will be seen that horse serum albumin behaves normally in alkaline or faintly acid solutions, but in 0.05 N HCl solution denaturation does not occur.

(1). 5 cc. of 1 per cent horse serum albumin (faintly acid), heated, flocculated without neutralization.

(2). 5 cc. of 1 per cent horse serum albumin + 0.5 cc. of 0.1 N HCl, heated, gave the same result as (1).

(3). 5 cc. of 1 per cent horse serum albumin + 1 cc. of 0.1 N HCl, heated, gave the same result as (1).

(4). 5 cc. of 1 per cent horse serum albumin + 2 cc. of 0.1 N HCl, heated, flocculated poorly without neutralization.

(5). 5 cc. of 1 per cent horse serum albumin + 5 cc. of 0.1 N HCl, heated, did not flocculate with or without neutralization. But the neutralized solution did flocculate on further heating, showing that the albumin was not denatured by the first heating.

(6). 5 cc. of 1 per cent horse serum albumin + 0.5 cc. of 0.1 N NaOH, heated, flocculated on neutralization.

(7). 5 cc. of 1 per cent horse serum albumin + 5 cc. of 0.1 N NaOH, heated, gave the same result as (6).

*Anomalous Behavior of Pigeon's Egg Albumin on Heating in Alkaline Solution.*—The following set of experiments shows that pigeon's egg albumin becomes insoluble when heated in acid or faintly alkaline solution, but in solutions containing moderate amounts of alkali the denatured product is soluble.

(1). 5 cc. of 1 per cent pigeon's egg albumin (faintly acid), heated, solution became opalescent without neutralization.

(2). 5 cc. of 1 per cent pigeon's egg albumin + 0.5 cc. of 0.1 N HCl, heated, solution became opalescent after neutralization.

(3). 5 cc. of 1 per cent pigeon's egg albumin + 5 cc. of 0.1 N HCl, heated, gave the same result as (2).

(4). 5 cc. of 1 per cent pigeon's egg albumin + 0.5 cc. of 0.1 N NaOH, heated, became opalescent after neutralization.

(5). 5 cc. of 1 per cent pigeon's egg albumin + 1 cc. of 0.1 N NaOH, heated, no opalescence with or without neutralization. The neutralized solution gave no opalescence on further heating, showing that the albumin had been changed by the first heating.

(6). 5 cc. of 1 per cent pigeon's egg albumin + 5 cc. of 0.1 N NaOH, heated, gave the same result as (5).

## DISCUSSION.

In our previous paper on the denaturation of proteins by dilute acids and alkalies we advanced the hypothesis that the fundamental reaction underlying denaturation is one of hydrolysis involving probably some especially labile linkages. Proteins which contain these labile linkages are subject to denaturation by dilute acids and alkalies, while those lacking these labile linkages are not subject to such denaturation.

If this hypothesis is true and if heat denaturation is the same process as denaturation by dilute acids and alkalies, it follows that those proteins which are subject to denaturation by one agent should be also subject to denaturation by the other. This is actually the case. Albumins, globulins, and hemoglobins are denatured by heat as well as by dilute acids and alkalies, while gelatin is denatured by neither. Albumoses and peptones which have lost the labile linkages through peptic digestion are not coagulable by heat nor are they denatured by dilute acids and alkalies.

In our previous paper we cautioned the use of acids and alkalies in isolating proteins on account of the danger of denaturation. The use of heat should be even more restricted, since heat causes denaturation so much more rapidly, and the protein is almost always denatured when heated for any length of time. The denatured protein differs, to be sure, only slightly from the original protein in the contents of most amino acids. But some amino acids which react with the phenol reagent—probably tyrosine and tryptophane—have been shown to be split off very readily. It is probable that some other amino acids which do not react with phenol reagent are also liberated when the protein is denatured.

It is well known that the non-protein blood filtrate prepared by heat coagulation contains more nitrogen than those prepared in the cold by means of protein precipitants in faintly acid solution. We have found the same to be true of non-protein chromogenic substances, and this is easily explained by the findings reported in this paper.

SUMMARY.

Evidence is adduced to show that the heat denaturation of proteins is the same process as the denaturation of proteins by dilute acids and alkalies at ordinary temperatures, and that the underlying reaction is one of hydrolysis.

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# SAPONINS.

## III. THE SAPOGENIN OCCURRING IN SAPINDUS SAPONARIA L. AND SAPINDUS MUKOROSSI UTILIS (TRABUTI).

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Our recent studies[1] on the sapogenin obtained from the saponin or saponins which occur in the shells of commercial soapnuts have resulted in its identification as hederagenin, the sapogenin which has been found in ivy leaves, *Hedera helix*. Owing to the importance of establishing definitely the botanical identity of the nuts which were used, a special effort has been made to obtain a quantity of the nuts of certain botanical origin. Through the very kind aid of Dr. John K. Small of the New York Botanical Gardens, the fruit of *Sapindus saponaria* L. at present growing on a plantation in Florida was secured. The shells of these nuts when worked up in the manner described in a former communication yielded a crystalline sapogenin which proved to be identical with hederagenin. This was confirmed by the preparation of the methyl ester and of the acetonyl derivative of this ester.

Since the nuts of this plant differed in general appearance from the commercial nuts which formed the material of our original investigations, we were led to believe that the commercial fruit in all probability had its origin in the plantations which occur in Algiers. Accordingly, through the kind efforts of Dr. Guggenheim of F. Hoffmann-La Roche and Co., of Basle, nuts were obtained from Algiers of the variety *Sapindus mukorossi utilis* (Trabuti) which owe their origin probably from plants of the variety *Sapindus mukorossi* Gaertn. which years ago had been brought from China and Japan. The shells of these nuts, which resembled closely the commercial variety with which we have

[1] Jacobs, W. A., *J. Biol. Chem.*, 1925, lxiii, 621, 631.

worked, have also yielded a crystalline sapogenin which was likewise identified as hederagenin. This was established further by conversion into its methyl ester and the acetonyl derivative of the latter.

The sapogenin occurring in *Sapindus mukorossi* has already been the subject of study by Winterstein and Blau[2] and later by Winterstein and Maxim[3] who, while describing physical properties which (the melting point of 319° reported by them differs but little from that of hederagenin, 332–334°) agree essentially with those of hederagenin, report, however, a formula based on their analytical results of $C_{18}H_{28}O_3$. If the substance in their hands was in reality hederagenin it is possible that the difficulty which has been frequently encountered in combustions with this substance may have been responsible for this error. The sapogenin which is obtained from *Hedera helix* as well as from *Sapindus saponaria* L. and *Sapindus mukorossi utilis* is hederagenin, $C_{31}H_{50}O_4$

EXPERIMENTAL.

*Sapindus saponaria L.*—1,570 gm. of soapnuts yielded 577 gm. of shells and 980 gm. of stones. The powdered shells, after extraction with 95 per cent alcohol and hydrolysis of the resulting extract as previously described, gave a crude crystalline sapogenin which, after digestion with acetone to remove colored impurities, weighed 25 gm. Recrystallized from alcohol it formed characteristic prisms and rhombic crystals which, after preliminary softening, melted to a turbid liquid at 332–334° which cleared a few degrees higher and when mixed with hederagenin showed no depression. The melting point of 327–329°, previously reported for hederagenin, had been determined with a thermometer which has since been found to be incorrect. In all other properties it resembled hederagenin.

$[\alpha]_D^{22} = +80°$ ($c = 1.025$ in pyridine).

$C_{31}H_{50}O_4$. Calculated. C 76.48, H 10.36.
Found. " 76.20, " 10.18.

The methyl ester prepared in the usual manner with methyl sulfate proved identical in all properties with hederagenin methyl

[2] Winterstein, E., and Blau, H., *Z. physiol. Chem.*, 1911, lxxv, 410.
[3] Winterstein, E., and Maxim, M., *Helv. Chim. Acta.*, 1919, ii, 195.

ester. It melted at 236–238° with preliminary softening and when mixed with hederagenin methyl ester no depression was noted.

*Air-Dry Substance.* Dried at 100° and 15 mm. over $H_2SO_4$.

$C_{32}H_{52}O_4 \cdot H_2O$. Calculated. $H_2O$ 3.47.
Found. " 3.00.

*Anhydrous Substance.*

$C_{32}H_{52}O_4$. Calculated. C 76.73, H 10.48.
Found. " 76.92, " 10.29.

The above ester yielded in the usual manner the characteristic acetonyl derivative which melted at 247–250° and showed no depression with the corresponding hederagenin derivative, and in other ways showed its identity with the acetonyl derivative of hederagenin methyl ester.

$C_{35}H_{56}O_4$. Calculated. C 77.71, H 10.44.
Found. " 77.88, " 10.14.

*Sapindus mukorossi utilis (Trabuti).*—1,640 gm. of soapnut yielded 1,138 gm. of shells and 500 gm. of nuts. The powdered shells yielded 55 gm. of crude sapogenin. The sapogenin, when recrystallized from alcohol, yielded the characteristic prisms and rhombs of hederagenin which melted at 332–334° after preliminary sintering.

$[\alpha]_D^{24} = +78°$ ($c = 1.015$ in pyridine).
$C_{31}H_{50}O_4$. Calculated. C 76.48, H 10.36.
Found. " 76.60, " 10.36.

The methyl ester prepared from the sapogenin melted at 236–239° and showed no depression when mixed with hederagenin methyl ester.

*Air-Dry Substance.* Dried at 100° and 15 mm. over $H_2SO_4$.

$C_{32}H_{52}O_4 \cdot H_2O$. Calculated. $H_2O$ 3.47.
Found. " 3.48.

*Anhydrous Substance.*

$C_{32}H_{52}O_4$. Calculated. C 76.73, H 10.48.
Found. " 76.50, " 10.44.

The above ester yielded again the acetonyl derivative characteristic of hederagenin methyl ester which melted at 249–252° and showed no depression when mixed with acetonyl hederagenin methyl ester.

$C_{35}H_{56}O_4$. Calculated. C 77.71, H 10.44.
Found. " 78.00, " 10.14.

# STROPHANTHIN.

## VII. THE DOUBLE BOND OF STROPHANTHIDIN.

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In a former communication[1] it was shown that the isomerization of strophanthidin to isostrophanthidin under the influence of alkali may occur without saponification of the lactone group. The carbonyl group was likewise found to play no apparent part in the reaction. The observation was also made that dihydrostrophanthidin prepared by the catalytic hydrogenation of strophanthidin was not isomerized when exposed to the action of alkali. The suggestion was offered at the time that the transformation of strophanthidin into its isomer may involve a shift in the double bond. More recent developments have substantiated this conclusion and have also permitted a clearer insight into the nature of this isomerization.

In the course of the study of the different substances encountered in our investigations with strophanthidin it has been generally noted that the lactone group can be readily saponified and that on reacidification the resulting hydroxy acid can be reconverted into the original lactone. For example, isostrophanthidin and its oxidation products, isostrophanthidic and the isostrophanthic acids, after saponification, are easily reconverted into the original lactone acids when reacidified with mineral acid. Similarly, dihydrostrophanthidin shows a like behavior and other instances might be noted. However, several exceptions to this rule have been encountered. The acid obtained by saponification of the ethylal of oxidodianhydrostrophanthidin shows, as previously recorded, no apparent tendency to lactonize again.[2] In more recent studies of the effect of alkali on dianhydrostrophanthi-

[1] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lxi, 387.

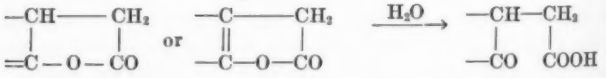
[2] Jacobs, W. A., and Collins, A. M., *J. Biol. Chem.*, 1924, lix, 713.



XUM

din itself similar observations bave been made. When saponified this substance yielded an acid, $C_{23}H_{30}O_5$, which in turn showed no tendency to revert to the original substance. The same acid was obtained also by hydrolysis of the ethylal of oxidodianhydrostrophanthidinic acid with dilute hydrochloric acid, a condition which should have caused a closing of the lactone group had there been such a tendency. In the attempt to perceive the cause for this departure from the behavior of the other substances mentioned above, which readily lactonized, two possibilities appeared. Either the formation of dianhydrostrophanthidin and its derivatives involved a change in the original molecule which rendered difficult ring closure when once the lactone group was opened, or, in the case of isostrophanthidin and dihydrostrophanthidin, reactions which involved the double bond of strophanthidin either by its change of position or hydrogenation, resulted in substances in which the hindrance to ring closure was removed. In the latter case strophanthidin itself after saponification should show but little tendency to lactonize again. Unfortunately the rapid isomerization of strophanthidin by alkali to isostrophanthidin rendered impossible determining this directly.

The nature of the double bond was, however, ascertained as follows: When the ethylal of oxidodihydrodianhydrostrophanthidin[3] was saponified, the resulting acid in contradistinction to its unhydrogenated analogue, the ethylal of oxidodianhydrostrophanthidinic acid, very readily reverted to the original lactone on acidification. In fact the tendency of this hydrogenated acid to lactonize was so great that ring closure occurred during attempts to recrystallize the acid. This observation is in complete harmony with the former experience with dihydrostrophanthidin and is readily explained by the assumption that strophanthidin and its anhydro derivatives are lactones of an enolized presumably γ-ketonic acid




which when once opened are prevented from closing again by the stability of the ketonic form. This condition disappears on

[3] Jacobs and Collins,[2] p. 729.

hydrogenation or when the double bond is shifted under the influence of alkali with the formation of the internal ester of a secondary alcohol. This conclusion was directly substantiated by the action of hydroxylamine on the ethylal of oxidodianhydrostrophanthidinic acid which yielded an oxime. On the contrary, when the sodium salt of the ethylal of oxidodihydrodianhydrostrophanthidinic acid was heated with hydroxylamine, it was recovered unchanged or rather as the lactone.

From these facts the general nature of the isomerization of strophanthidin to isostrophanthidin appears established, but the position of the double bond in the latter can be determined only by further work. In strophanthidin itself, if a $\gamma$ - lactone, the choice for the moment between a $\Delta_{\beta-\gamma}$-crotonic lactone and a double bond outside of the lactone ring is difficult. The behavior of strophanthidin and its anhydro derivatives towards Tollens' reagent suggests that the double bond may be perhaps within the lactone ring in position $\beta$–$\gamma$ to the C = O group. These substances in dilute alcoholic solution reduce the reagent fairly readily although not immediately as described by Thiele[4] for the $\Delta_{\alpha-\beta}$- and $\Delta_{\beta-\gamma}$-crotonic lactones. It is questionable, however, how conclusive such tests can be with such a complicated molecule as strophanthidin. On the other hand, isostrophanthidin and dihydrostrophanthidin act far more slowly on this reagent. In the case of isostrophanthidin the double bond, if still present, appears to be outside of the lactone ring. We hope, when additional facts are available, to coordinate these results with those obtained by previous oxidation experiments.

It is apparent that when dianhydrostrophanthidin and its ethylal are saponified the simultaneous shift in the double bond such as results in the formation of isostrophanthidin from strophanthidin has been prevented. From former work we have assumed in dianhydrostrophanthidin conjugated double bonds within a 6-membered carbon ring. It is possible that the formation of these double bonds has involved the removal of hydrogen atoms originally present in strophanthidin which permitted the shift in the double bond to form isostrophanthidin. In dianhydrostrophanthidin this shift has become no longer possible.

[4] Thiele, J., *Ann. Chem.*, 1901, cccxix, 144.

In a former paper[5] the lactone acid, $C_{23}H_{30}O_7$, obtained by oxidation of strophanthidin in acetone solution with permanganate, was described which after saponification could be further oxidized to a ketonic acid which no longer formed a lactone. In view of the above results it appeared necessary to reconsider the results there presented in order to determine whether the ketonic group in this ketonic acid was in reality the result of oxidation or whether it was originally present in the saponified lactone acid, $C_{23}H_{30}O_7$, before oxidation. To determine this point the latter acid after saponification was treated with hydroxylamine. The unchanged lactone acid, $C_{23}H_{30}O_7$, was recovered after acidification and no evidence of the formation of an oxime could be obtained. The original conclusion is therefore justified that the ketonic group in the keto acid was produced by oxidation of a secondary alcoholic group produced by opening up the lactone ring. It is apparent, therefore, that the production of the lactone acid, $C_{23}H_{30}O_7$, by the oxidation of strophanthidin, has also involved the double bond. The exact mechanism must be a subject for further investigation.

## EXPERIMENTAL.

*Dianhydrostrophanthidinic Acid.*—5 gm. of dianhydrostrophanthidin were refluxed for 45 minutes with a mixture of 500 cc. of 0.1 N NaOH and 125 cc. of alcohol. The chilled solution was acidified with acetic acid, causing the separation of a flocculent precipitate. After collection with water the amorphous material was made to crystallize by digestion under a small volume of dry acetone. The collected substance was suspended in 50 per cent alcohol and dissolved by the addition of a slight excess of ammonia. On careful acidification with acetic acid the acid slowly deposited as compact aggregates of stout crystals and rosettes of thinner needles and leaflets which were collected with 50 per cent alcohol. The substance is completely soluble in dilute ammonia and melts at 215–218° after preliminary softening. In sulfuric acid it dissolves with a yellowish brown color which deepens to a red-brown.

$[\alpha]_D^{24} = -176°$ ($c = 0.510$ in a mixture of equal volumes of chloroform and alcohol).

[5] Jacobs, W.A., *J. Biol. Chem.*, 1923, lvii, 556.

The substance showed no apparent mutarotation under these conditions.

*Air-Dry Substance.* Dried at 100° and 15 mm. over $H_2SO_4$.

$C_{23}H_{30}O_8 \cdot \frac{1}{2}H_2O$. Calculated. $H_2O$ 2.28.
Found. " 2.02.

*Anhydrous Substance.*

$C_{23}H_{30}O_8$. Calculated. C 71.46, H 7.83.
Found. " 71.39, " 7.84.

A Zeisel determination was negative.

Attempts to close the lactone group resulted either in recovery of unchanged material or of amorphous alteration products which, however, were still soluble in alkali. An attempt was made with negative results to convert the acid into the sparingly soluble ethylal of oxidodianhydrostrophanthidin by warming in absolute alcoholic hydrochloric acid which while ethylating the carbonyl group should also have caused lactonization if there had been any such tendency.

The same acid was obtained as follows: 1 gm. of the ethylal of oxidodianhydrostrophanthidinic acid was refluxed for 45 minutes in 50 cc. of 50 per cent alcohol, containing 2 per cent of HCl. When poured into water a voluminous precipitate formed which was collected. The amorphous substance was digested with a small volume of dilute ammonia, leaving an appreciable amount of an apparently neutral amorphous alteration product. The filtrate was treated with a half volume of alcohol and then acidified with acetic acid. Bundles of fine needles separated which were completely soluble in ammonia and melted at 217–219° and gave no depression when mixed with the acid prepared as above from dianhydrostrophanthidin.

$[\alpha]_D^4 = -178°$ ($c = 0.505$ in 1:1 $CHCl_3$ + alcohol).

*Anhydrous Substance.*

$C_{23}H_{30}O_8$. Calculated. C 71.46, H 7.83.
Found. " 71.92, " 7.79.

*Oxime of the Ethylal of Oxidodianhydrostrophanthidinic Acid.*—1.2 gm. of the acid obtained by saponification of the ethylal of oxidodianhydrostrophanthidin were refluxed in 40 cc. of alcohol for 2 hours with 0.8 gm. of hydroxylamine hydrochloride and 3.2 gm. of sodium acetate. The mixture, when poured into

water slightly acidified with acetic acid, gave a voluminous precipitate which changed to needles. Recrystallized from dilute alcohol, it formed needles which melted at 170–172° with effervescence.

*Air-Dry Substance.* Dried at 100° and 15 mm. over $H_2SO_4$.

$C_{25}H_{35}O_5N \cdot \frac{1}{2}H_2O$. Calculated. $H_2O$ 2.05.
Found. " 1.60.

*Anhydrous Substance.*

$C_{25}H_{35}O_5N$. Calculated. C 69.88, H 8.22, N 3.26, $OC_2H_5$ 10.50.
Found. " 69.99, " 8.29, " 3.27, " 9.81.

*Saponification of the Ethylal of Oxidodihydrodianhydrostrophanthidin.*—1 gm. of the ethylal was refluxed for 1 hour with 100 cc. of alcohol and 75 cc. of 0.1 N NaOH. After concentration under diminished pressure to small bulk the mixture was carefully acidified with acetic acid, causing the separation of a voluminous precipitate which was collected at once with water. It was dissolved in a small volume of warm alcohol and diluted with an equal volume of warm water. A thick mass of needles separated on cooling. After collection, the substance was found to be no longer completely soluble in dilute KOH. It was digested in the cold with a slight excess of dilute KOH solution and the insoluble portion collected. When this neutral substance was recrystallized from dilute alcohol it formed delicate needles which melted at 207–210° with preliminary softening and showed no depression when mixed with the original lactone.

$$[\alpha]_D = -92° \ (c = 1.020 \text{ in } CHCl_3).$$

When the alkaline mother liquor from the above neutral substance was treated with one-half volume of alcohol and carefully acidified in the cold with acetic acid, the acid separated at first as a gelatinous precipitate which changed on rubbing to fine needles which were collected with 25 per cent alcohol. The acid melted at 197–202° and was completely soluble in dilute alkali, but when gently warmed with dilute acid it rapidly changed to the crystalline lactone.

The great tendency to lactone formation was further demonstrated in the following unsuccessful attempt to prepare an oxime: 1.4 gm. of the ethylal of oxidodihydrodianhydrostrophanthidin were saponified in a mixture of 140 cc. of alcohol and 105 cc. of

0.1 N NaOH and, after careful neutralization to phenolphthalein with dilute acetic acid, the mixture was concentrated under diminished pressure to dryness. The salts were dissolved in 50 cc. of 50 per cent alcohol and after the addition of 0.5 gm. of hydroxylamine hydrochloride and 2 gm. of sodium acetate, the mixture was refluxed. After about ½ hour boiling, a mass of needles separated which was collected with 50 per cent alcohol. After recrystallization from alcohol the substance melted at 206–210° and showed no depression when mixed with the original ethylal. The substance contained no nitrogen. On working up the original mother liquor small amounts of the same substance were recovered.

| | | |
|---|---|---|
| $C_{25}H_{34}O_4$. | Calculated. | C 75.33, H 8.61. |
| | Found. | " 75.37, " 8.43. |

# THE EFFECT OF HEAT ON THE SOLUBILITY OF THE CALCIUM AND PHOSPHORUS COMPOUNDS IN MILK.

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## INTRODUCTION.

In reviewing the literature on the effect of heat on milk, it appears that considerable difference of opinion still exists relative to the probable effect of heat on the chemical and physical properties of the mineral salts of milk.

Some observers claim that the heating of milk causes part of the soluble compounds containing calcium and phosphorus to change to an insoluble condition and be precipitated, while others claim that there is no noticeable change in the solubility of the compounds. Not only is this found in the strictly chemical investigations of milk, but in the nutrition experiments conducted by Lane-Claypon (1916), Daniels and Loughlin (1920), and others on the nutritive value of raw and boiled milks.

If a soluble calcium or phosphorus deficiency exists in heated milk, the logical way to prove it is by chemical analysis, but on turning to the investigations along chemical lines, we are confronted with marked differences in data and opinions with regard to the influence of heat on the composition of the milk. The probable reason for this undoubtedly lies in a lack of uniformity of methods and milks used. Some used whole milk, others skim milk. The age of the milk is of especial importance, the older milk having the higher acid content, due to bacterial action. In filtration experiments carried on in connection with this investigation, it was found that as the acidity of the milk in the filtering apparatus increased, due to natural souring, the amount of CaO and $P_2O_5$ in the filtrate gradually approached a value close to that of the total CaO and $P_2O_5$ content of the fresh skim milk before starting to filter.



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## *Methods.*

### *Treatment of Milk.*

Standardization of methods and elimination, as far as possible, of all factors which might tend to produce a change in the milk, other than heat, are of prime importance, so as to have the samples on a uniform basis for comparison. With this in mind and to overcome the objections previously discussed, the following general methods were carried out.

In all of the experiments performed, only fresh, raw, skim milk was used. As soon as obtained, it was thoroughly mixed and divided into two portions, one part going to the cooler to prevent chemical change and the other taken to the laboratory for the beginning of the experiment. Skim milk was used because the fat of whole milk would clog the pores of the Pasteur-Chamberland filters used and also, in the second part of the work, because of the construction of the supercentrifuge.

In the work with the Pasteur-Chamberland filters, the milk was heated in a water bath in an open container. No account was taken of the loss in volume due to evaporation. In the work with the supercentrifuge, the milk was heated under a reflux condenser, thus preventing a loss due to evaporation. In all cases, as soon as the desired temperature for holding the milk had been reached, the temperature of the water in the water bath was brought down to that of the milk by the addition of cold water. All samples were maintained at the holding temperature for 30 minutes. At the end of that time, the samples heated in an open container were cooled as soon as possible under the cold water tap. Those samples in the second part of the work were cooled in the same way except that the container was closed. In all cases, the milk container in the water bath rested on a wire support, thereby protecting the milk at the bottom of the container from the more direct heat near the source of application. Up to the time of cooling, the milk was agitated only enough to insure an even distribution of heat. On cooling, however, the milk container was continuously agitated while under the tap to hasten the cooling process. This continued until the milk in the container had attained a temperature of 20°C.

It can readily be seen that the time of heating to bring the milk to the holding temperature increased as the temperature of the holding became higher. The intensity of the heat applied was regulated as much as possible so that the increased length of time to attain the higher temperature would have a definite ratio. For instance, it would take 20 minutes to heat the milk to 180°F., 25 minutes to attain 190°F., 30 minutes to reach 200°F., etc. It also holds that the higher the holding temperature, the longer the time necessary for cooling.

*Filtration Methods.*

Many of our ideas concerning the state in which the various constituents of milk occur, are the result of filtration studies in which the milk was filtered through earthenware filters. The variety of individual filters used, influenced the results. Among the more important studies of this type, that of Rupp (1913) is of special interest. He obtained a serum by filtering milk through "a porous clay cell of a galvanic element," using suction. He concluded that pasteurization for 30 minutes at 68°C. did not affect the content of soluble calcium, magnesium, and phosphorus compounds in the serum.

Probably the most extensive studies of the condition of the various compounds in milk are those of Van Slyke and Bosworth (1914). Others who have worked on the effect of heat on milk are Palmer (1921–22), Palmer and Scott (1919), Jackson and Rothera (1914), and Flohil (1911). Grosser (1919) has shown that raw milk gave an ultrafiltrate much richer in lime than did boiled milk. In boiling, the Ca is bound to the milk colloid and remains with the latter on the ultrafilter.

The methods of study most frequently resorted to are dialysis and filtration. Dialysis studies at best are unsatisfactory when working with such a complex material as milk because of the danger of shifting the original state of equilibrium. On this account, filtration methods seem more desirable. Much of the work already reported is difficult to interpret because of the apparent unknown variations in the porosity of the filters used. It therefore seemed desirable to attempt filtration studies, using filters the relative porosity of which was known.

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Briefly stated, the process consisted in putting the milk into a tubular chamber surrounding a Pasteur-Chamberland filtering tube. Pressure amounting to 50 to 60 pounds per square inch was then applied by means of a pump, which forced air into the chamber containing the milk and caused the soluble portion of the milk to pass through the walls of the filter from the outside to the inside of the filter tube. Any pressure greater than that mentioned did not seem to increase the speed of filtration.

The insoluble residue accumulated on the outside surface of the filter tube, from which it was removed by light scraping with a stiff brush and the use of running water forced from the inside to the outside of the tube. Since the filter has the power of adsorbing some of the constituents of the serum until a volume of 50 to 75 cc. has passed through, this amount should be discarded. After this, the filtered serum was constant in composition. This was the opinion of Rupp (1913), and it was verified in this work by trials, the results of which are not given in the data here reported.

Before being placed in the apparatus for filtration, the milk was cooled to 40°F. to prevent souring during the process of filtration. No antiseptic for preserving was ever used. The filtering apparatus was then placed in the cooler where a constant temperature of approximately 40°F. was maintained. New candles were used two or three times before using them for experimental data. In cleaning, the directions as given by the company manufacturing the candles were followed carefully.

*Centrifugal Methods.*

Milk is an aqueous solution of crystalloids (salts and milk sugar) which contains the colloids, casein and albumin, and also an emulsion of fat. If, according to Grosser (1919), the lime is attached more firmly to the milk colloids in boiled milk than in unboiled milk, then, provided a suitable means of separating the milk colloids from the aqueous solution of crystalloids is found, the lime and phosphorus content of the aqueous solution of crystalloids should be less in boiled milk than in raw milk from the same source.

Therefore, in addition to the experimental methods already described, it was decided to make this separation by means of a high speed centrifuge. The supercentrifuge was so constructed

that the milk could be passed continuously through the rotating member, where it was subjected to a force approximately 40,000 times that of the force of gravity and was then continuously discharged.

The samples of milk were obtained and prepared as described previously. Although 120°F. is the temperature of minimum viscosity for milk, it was thought best to make the centrifuging temperature for the raw and heated skim milk samples 70°F. which is normal room temperature. 500 cc. of each kind of milk were centrifuged each time. After about 300 cc. of the liquid had entered the bowl, the bowl was full enough to overflow from the discharge holes in the top into the cover. The liquid then flowed from the cover into a suitable dry container.

At the end of the operation, the machine was allowed to stop, the bowl was removed, and the deposited solid matter obtained by scraping the contents into a tared porcelain dish. The solid matter was dried and incinerated and the weight of the ash obtained in each case. The ash was in turn analyzed for the weight of lime (CaO) and phosphorus ($P_2O_5$) which it contained.

The liquid discharged from the cover (which will hereafter be called the filtrate) was measured for the volume and 100 gm. of it were ashed and analyzed for the weight of CaO and $P_2O_5$ which it contained. The volume of the filtrate was in every case approximately 190 cc.

### *Analytical Methods.*

The phosphorus analyses were made as recommended by the Association of Official Agricultural Chemists.

The calcium analyses were made according to McCrudden (1911–12).

## RESULTS.

### *Filtration Experiments.*

In order to test the variability of individual candles, a number of tests were made in which duplicate samples of raw skim milk were passed through different filters. From the results, as shown in Table I, it was computed that there was a possible variation between the candles of 0.0042 per cent in the determination of Ca

in the filtrate, and of 0.0038 per cent in the determination of $P_2O_5$ from the same source.

Experiments were then carried on to see if the difference in the percentage of Ca and $P_2O_5$ in the filtrate from the raw and heated samples from the same source would exceed this value. The candles were used interchangeably, no means being taken to distinguish one from the other in succeeding experiments. The

TABLE I.

*Comparison of Candles on Duplicate Runs of Raw Skim Milk.*

| Run No. | Candle. | Ca | Difference in Ca. | $P_2O_5$ | Difference in $P_2O_5$. |
|---|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* | *per cent* |
| 1 | 1 | 0.0265 | | 0.0627 | |
| | 2 | 0.0264 | −0.0001 | 0.0606 | −0.0021 |
| 2 | 1 | 0.0253 | | 0.0674 | |
| | 2 | 0.0249 | −0.0004 | 0.0656 | −0.0018 |
| 1 | 3 | 0.0280 | | 0.0669 | |
| | 4 | 0.0318 | +0.0038 | 0.0686 | +0.0017 |
| 1 | 5 | 0.0215 | | 0.0646 | |
| | 6 | 0.0215 | 0.0000 | 0.0633 | −0.0013 |
| 2 | 5 | 0.0254 | | 0.0612 | |
| | 6 | 0.0254 | 0.0000 | 0.0609 | −0.0003 |
| Average..... | | | +0.0007 | | −0.0007 |

average difference between the percentage of Ca and $P_2O_5$ in the filtrate from the raw and heated milk was obtained by determining the sum of the positive and the negative differences and dividing that difference by the total number of determinations made at that particular temperature. For example, at 140°F. the loss in the Ca content of the filtrate from the skim milk, as a result of heating, exceeded the gain by an average of 0.0032 per cent for six determinations. The loss in the $P_2O_5$ content of the filtrates from the skim milk, due to heating, exceeded the gain by an average of 0.0018 per cent for six determinations.

While the results obtained with the samples heated to tempera-

tures of 140–160°F. yielded results which perhaps fall within the experimental error, the experiments with the higher temperatures gave more marked differences. The greatest variation between any two of the candles used, when duplicates of raw skim milk were filtered, was 0.0041 per cent in the case of Ca and 0.0038 per cent in the case of $P_2O_5$. It appears that the difference in the percentage composition of Ca in the filtrate from raw and heated skim milk, and of $P_2O_5$ from the same source and under the same experimental conditions, does not exceed the possible experimental error in the candles unless the milk has been heated for 30 minutes to 170°F. or above. Even at these temperatures, the difference is small.

TABLE II.

*Summary of Losses of Soluble Ca and $P_2O_5$ Due to Heating (Filtration Method).*

| Temperature to which heated for 30 min. | Average loss in Ca in filtrate. | Average loss in $P_2O_5$ in filtrate. |
|---|---|---|
| °F. | *per cent* | *per cent* |
| 140 | 0.0032 | 0.0018 |
| 150 | 0.0011 | 0.0024 |
| 160 | 0.0020 | 0.0016 |
| 170 | 0.0053 | 0.0059 |
| 180 | 0.0039 | 0.0042 |

It should be taken into consideration, however, that for the temperatures ranging from 140–160°F. the *majority* of the results, from which the averages in Table II were taken, showed a small loss in percentage of soluble Ca and $P_2O_5$ over that found in the raw skim milk. For the temperatures of 170°F. and 180°F. the results in *every case* showed a small loss of both compounds in the filtrate, due to the heating of the skim milk. The difference at the higher temperatures persists in the results in spite of the fact that the concentration of the soluble salts in the serum would tend to increase, due to evaporation of the moisture in the heating process.

The conclusions that must be drawn from these results are that the changes from soluble to insoluble salts containing calcium and phosphorus are small and that, even for the higher temperatures employed, it but slightly exceeds the possible experimental error.

In the present study, the average percentage of Ca of samples of the filtrate from raw skim milk was 0.0272. In terms of CaO, this is an average of 0.0381 per cent. Since the average CaO content of the skim milk used was 0.16 per cent, this means that an average of 23.81 per cent of the CaO content of the skim milk remained in the filtrate or was in true solution. The lowest analysis showed 0.0206 per cent Ca (0.0298 per cent CaO) and the highest analysis yielded 0.0385 per cent Ca (0.0539 per cent CaO).

*Centrifugal Experiments.*

The average gain (+) or loss (−) in CaO content of the filtrate in terms of percentage was obtained by getting the average loss

TABLE III.

*Summary of Results (Centrifugal Method).*

| Temperature to which heated for 30 min. | Average gain or loss in CaO. | | Average gain or loss in $P_2O_5$. | | Average gain or loss in ash. | |
|---|---|---|---|---|---|---|
| | Filtrate. | Precipitate. | Filtrate. | Precipitate. | Filtrate. | Precipitate. |
| °F. | *per cent* | *gm.* | *per cent* | *gm.* | *gm.* | *gm.* |
| Raw (checks). | +0.88 | −0.0126 | +1.36 | −0.0007 | −0.0075 | −0.0571 |
| 150 | +0.19 | −0.0033 | −0.78 | −0.0009 | −0.0035 | −0.0389 |
| 160 | −0.35 | +0.0075 | −2.47 | +0.0131 | −0.0030 | +0.0203 |
| 170 | −1.59 | +0.0072 | −2.56 | +0.0085 | −0.0119 | +0.0025 |
| 180 | −4.34 | +0.0510 | −5.68 | +0.0033 | −0.0240 | +0.1149 |
| 190 | −7.58 | +0.0903 | −6.92 | +0.0145 | −0.0310 | +0.2164 |
| 200 | −7.04 | +0.1003 | −8.77 | +0.0201 | −0.0394 | +0.2688 |
| 212 | −9.75 | +0.1246 | −9.53 | +0.0218 | −0.0419 | +0.2954 |

or gain in gm. of CaO in the filtrates from the raw and heated skim milk and then calculating what percentage this figure was of 0.16. The average CaO content of the skim milk analyzed was 0.16 per cent. The average gain (+) or loss (−) in $P_2O_5$ in the filtrate in terms of percentage was determined in like manner, 0.2117 being the average percentage of $P_2O_5$ content of the skim milk analyzed. These results are summarized in Table III.

The amount of calcium, phosphorus, and ash in the filtrates and precipitates varied considerably in the different runs. This was due in part to the speed of the centrifuge. Some runs were made

at a speed of 38,000 R.P.M., others at 40,000 R.P.M., and also at speeds between these. To get the R.P.M. of the bowl, a stop-watch was always used. If it was found that the heated portion of the skim milk was being centrifuged at a speed less than 40,000 R.P.M., that speed was duplicated as nearly as possible for the raw skim milk from the same source.

Inspection of Table III reveals the fact that with an increase in temperature, the amount of soluble CaO and $P_2O_5$ in the filtrate decreases gradually from the amount found in the filtrate from the raw skim milk from the same source. The precipitate from these samples showed a steady gain in the weight of these substances from the same source. Furthermore, as the temperature of heating increased, the weight of the ash from 100 gm. of the filtrate from the heated milk decreased. On the other hand, with an increase in the heating temperature, the amount of solids thrown out by the centrifuge gradually increased over the amount deposited by the centrifuge from the raw skim milk.

## SUMMARY.

Fresh skim milk was heated to various temperatures in order to study the effect of heat on the solubility of the calcium and phosphorus content. These studies were made with the aid of Pasteur-Chamberland filters and a high speed supercentrifuge.

It appears from the results that there is a loss in the soluble calcium and phosphorus contents of the skim milk due to heat and that the amount of the loss depends upon the temperature to which the milk has been heated.

The results from the methods employed indicate that definitely measurable amounts of these substances are removed from solution in milks heated to 170°F. or above.

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# A NOTE ON THE INFLUENCE OF THE DIET ON THE SYNTHESIS OF HIPPURIC ACID.

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In a recent paper Abderhalden and Wertheimer[1] reported experiments in which they found that rabbits on a diet of oats were able to synthesize hippuric acid more readily than rabbits on a diet of green vegetables. They believed that the reaction of the diet was responsible for this difference. The diet of oats, having an acid ash and producing an acid urine, was considered favorable for the synthesis of hippuric acid; while the diet of green vegetables, having an alkaline ash and producing an alkaline urine, was considered unfavorable. They stated their conclusions as follows:

"Wir glauben daher sagen zu können, dass es zum ersten Mal gelungen ist, eine wichtige Synthese in ihrem quantitativen Ablauf im Organismus zu beeinflussen, und zwar auf ganz natürliche Weise, indem wir durch eine verschiedenartige Ernährung die Bedingungen zur Synthese in den massgebenden Zellen verändern. Bei Hafernahrung sind diese für die Synthese optimal, bei Grünfutternahrung minimal, durch gemischte Nahrung werden wir Bedingungen shaffen können, die zwischen diesen beiden Extremen liegen. Wir haben es also geradezu in der Hand, den Ablauf einer Synthese im intakten Organismus durch einfache Massnahmen zu regulieren."

It is not to be doubted that the type of diet may influence the synthesis of hippuric acid. Nevertheless, the incredibly low values obtained by Abderhalden and Wertheimer for the rabbits on the green vegetable diet demanded further explanation than that offered by these investigators. Such an explanation was provided by a careful examination of their analytical procedure.

[1] Abderhalden, E., and Wertheimer, E., *Arch. ges. Physiol.*, 1924, ccvi, 460.

The method used for the determination of hippuric acid was that of Snapper and Laqueur.[2] This is based upon the extraction of hippuric acid from 50 cc. of urine, acidified with 0.3 cc. of concentrated HCl, by six portions of ethyl acetate. It occurred to us that 0.3 cc. of concentrated HCl might be inadequate for the complete extraction of hippuric acid from alkaline urines such

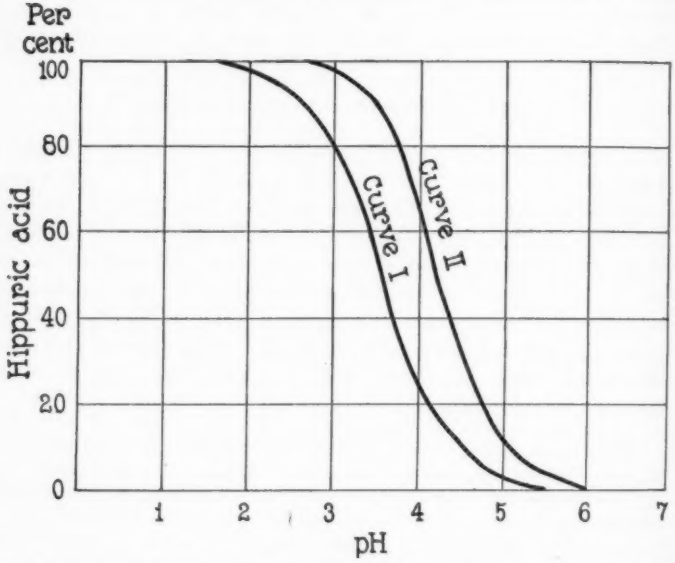


CHART 1. Curve I. The influence of the reaction of a solution on the percentage of the total hippurate (acid and salt) which is present as hippuric acid.

Curve II. The influence of the reaction of a solution on the percentage of the total hippurate which may be extracted by the method of Snapper and Laqueur.

as those obtained from rabbits on a green vegetable diet. This possibility was verified by experiments which will be reported in this paper.

In a solution containing hippuric acid and its salt, the ratio of the concentrations of the acid and of the salt are dependent upon

[2] Snapper, I., and Laqueur, E., *Biochem. Z.*, 1924, cxlv, 32.

the hydrogen ion concentration of the solution. This relation is best shown by Curve I (Chart 1) which was obtained as follows:

Since,

$$(H^+) = K \times \frac{\text{acid}}{\lambda \text{ salt}}$$

Then,

$$(H^+) = K \times \frac{HA}{\lambda (T - HA)}$$

where $(H^+)$ is the hydrogen ion concentration, $K$ the dissociation constant for hippuric acid, $\lambda$ the percentage dissociation of sodium hippurate, $T$ the mols of total hippurate (acid and salt), and HA the mols of acid.

Rearranging,

$$\frac{HA}{T} = \frac{(H^+) \times \lambda}{K + (H^+) \times \lambda}$$

The percentage of hippuric acid present is $\frac{HA \times 100}{T}$. Therefore,

$$\text{Percentage of hippuric acid} = \frac{(H^+) \times \lambda \times 100}{K + (H^+) \times \lambda}$$

The dissociation constant for hippuric acid is $2.2 \times 10^{-4}$. It was assumed that sodium hippurate in approximately 0.01 molar concentration was 85 per cent dissociated. By substituting values for $(H^+)$ from $1 \times 10^{-2}$ to $1 \times 10^{-6}$, the corresponding values for the percentage of hippuric acid present were calculated, and were plotted against pH. For example, at pH 4.0, $(H^+) = 1 \times 10^{-4}$, and the percentage of hippuric acid is

$$\frac{1 \times 10^{-4} \times 0.85 \times 100}{2.2 \times 10^{-4} + 1 \times 10^{-4} \times 0.85} = 27.9 \text{ per cent}$$

In other words, in a urine containing hippuric acid and its salt, only 27.9 per cent of the total hippurate is present as hippuric acid at pH 4.0. The curve also shows that practically all of the

hippurate would be present as the salt at pH 5.0 and as the acid at pH 2.0. It is evident that a urine might be made acid to litmus (*i.e.*, with pH 5.5 to 6.5) without converting more than traces of the salt to the acid. This curve is important because the percentage of hippuric acid which may be extracted from a urine depends upon the percentage of hippuric acid which is present and not upon the total hippurate (acid and salt).

A second curve was plotted showing the percentage of hippuric acid which may be extracted from urine at different hydrogen ion concentrations by the method of Snapper and Laqueur (Curve II, Chart 1). There are three factors affecting the extraction of hippuric acid from urine which make the second curve different from the first. These factors are (1) an increase in the pH of the urine, (2) a shift of sodium hippurate to hippuric acid, and (3) the distribution ratio of hippuric acid between ethyl acetate and urine. As hippuric acid is removed from urine, the pH of the urine increases and the percentage of hippuric acid present decreases (Curve I). At the same time, some sodium hippurate is converted into hippuric acid, the extent of this change depending upon the change in pH. Therefore, the amount of hippuric acid which is removed by six extractions with ethyl acetate is greater than the quantity originally present in the urine as acid. In plotting Curve II, the calculations were made on the basis that the pH remained constant and, therefore, that the percentage of hippuric acid present remained the same. Since the percentage of acid present actually decreased during the extraction, the curve, in this respect, gives values which are somewhat high. Snapper and Laqueur[2] found that the distribution ratio of hippuric acid between ethyl acetate and water is such that 60 per cent of the hippuric acid is removed from an aqueous solution by a single extraction with ethyl acetate. This distribution ratio was used in the calculations for Curve II. Since it is probable that more than 60 per cent of the hippuric acid present in an acid urine is removed by a single extraction with ethyl acetate, the curve, in this respect, gives values which are somewhat low. However, neither the change in pH nor the value of the distribution ratio materially affects the curve and it will be shown later that Curve II agrees satisfactorily with the experimental results. The curve was obtained as follows:

The percentage of hippuric acid present in a solution containing hippuric acid and sodium hippurate is given by the expression

$$\frac{(H^+) \times \lambda \times 100}{K + (H^+) \times \lambda}$$

Therefore, the percentage of hippuric acid ($P_1$) removed by a single extraction with ethyl acetate is

$$0.6 \left( \frac{(H^+) \times \lambda \times 100}{K + (H^+) \times \lambda} \right)$$

If $(H^+) = 1 \times 10^{-4}$, then

$$P_1 = \frac{1 \times 10^{-4} \times 0.85 \times 100 \times 0.6}{2.2 \times 10^{-4} + 1 \times 10^{-4} \times 0.85} = 16.7 \text{ per cent}$$

The percentage of hippuric acid removed by the second extraction ($P_2$) is 0.167 (100 per cent − 16.7 per cent) or $P_2$ = 13.9 per cent.

Similarly,

$P_3$ = 0.167 (83.3 per cent − 13.9 per cent) = 11.6 per cent
$P_4$ = 0.167 (69.4 " " − 11.6 " " ) = 9.7 " "
$P_5$ = 0.167 (57.8 " " − 9.7 " " ) = 8.0 " "
$P_6$ = 0.167 (48.1 " " − 8.0 " " ) = 6.7 " "
Total percentage extracted at pH 4.0 = 66.6 " "

In other words, *the quantitative analysis of a urine at pH 4.0 by the method of Snapper and Laqueur would result in a determination of only 67 per cent of the hippurate actually present.* Curve II shows that the reaction of the urine must be at least pH 3.0 in order to effect complete extraction of the hippurate present. These theoretical considerations show that variable and incorrect results would be obtained whenever urines were not made sufficiently acid before analysis.

This possibility of using too little acid when determining hippuric acid in alkaline urines by the method of Snapper and Laqueur was also tested experimentally. Rabbits, which were allowed to eat cabbage and carrots freely, were fed 1.5 gm. of benzoic acid (as the sodium salt) and the urine was collected for the following 10 to 16 hour period. The urines were pre-

XUM

served with chloroform. The method of Snapper and Laqueur was used for the determination of hippuric acid, but in each case a second determination was made in which 1 to 2 cc. of concentrated HCl were added to the urine before extraction with ethyl acetate. The pH of the urine, before and after the addition of acid, was determined colorimetrically. The results of these experiments are given in Table I. One experiment on an oat-fed rabbit is included in the table.

TABLE I.

*Influence of Reaction (pH) on the Extraction of Hippuric Acid from Urine by the Method of Snapper and Laqueur.*

| Experiment No. | Rabbit No. | Volume of urine. | Reaction of urine. | Hippuric acid (calculated as benzoic acid) using | | | Reaction of 50 cc. urine + 0.3 cc. concentrated HCl. | Extraction of hippuric acid (from curve).* | Diet. | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 to 2 cc. concentrated HCl. | 0.3 cc. concentrated HCl. | | | | | | |
| | | *cc.* | *pH* | *gm.* | *gm.* | *per cent* | *pH* | *per cent* | | | |
| 1 | 3 | 83 | 5.5 | | 1.023 | | | | Oat. | | |
| 2 | 3 | 200 | 8.0 | 1.025 | 0.084 | 8 | 5.0 | 12 | Cabbage and carrot. | | |
| 3 | 3 | 280 | 8.0 | 0.940 | 0.114 | 15 | 5.0 | 12 | " | " | " |
| 4 | 1 | 175 | 8.0 | 1.044 | 0.235 | 22 | 4.5 | 34 | " | " | " |
| 5 | 3 | 110 | 8.0 | 0.986 | 0.505 | 51 | 4.0 | 67 | " | " | " |
| 6 | 2 | 200 | 8.0 | 1.148 | 0.585 | 51 | 4.2 | 51 | " | " | " |
| 7 | 2 | 150 | 8.0 | 1.051 | 0.692 | 66 | 3.5 | 91 | " | " | " |
| 8 | 3 | 260 | 8.0 | 1.165 | 1.100 | 94 | 3.5 | 91 | " | " | " |
| 9 | 1 | 200 | 7.0 | 1.215 | 1.160 | 95 | 3.0 | 98 | " | " | " |

* The values in this column were read from Curve II (Chart 1) and correspond to the pH values in the preceding column.

In seven of the eight experiments on the green vegetable diet the urines were alkaline; the other was neutral. *In not one of these eight experiments was there complete extraction of the hippurate when 0.3 cc. of concentrated HCl was used to acidify the urine.* It is apparent from the table that the percentage of extraction depended upon the pH of the urine extracted. Thus, in Experiment 2, 8 per cent of the total hippurate was extracted from a urine with a pH of 5.0, while 95 per cent was extracted from a

urine with a pH of 3.0. The values in the next to the last column of Table I were obtained from Curve II and correspond to the pH values in the preceding column. They represent the theoretical percentage of extraction of hippurate from urines in which the pH is known. The agreement of these values with the actual values was considered satisfactory in view of the fact that the pH determinations were only approximate.

When the procedure used by Abderhalden and Wertheimer was followed as closely as was possible, results of the same type as their results were obtained. It is obvious that these results were incorrect. *The results obtained when the urines were made sufficiently acid before extraction showed that there was no apparent diminution in the ability of rabbits on a green vegetable diet to synthesize hippuric acid after the administration of sodium benzoate.* Rabbit 3 excreted as much combined benzoic acid after 8 days on the green vegetable diet (Experiment 8) as it did on the oat diet (Experiment 1).

In Experiment 5[3] 125 cc. of alkaline urine were obtained 20 hours after the administration of 1.5 gm. of benzoic acid. Assuming that 70 to 75 per cent of the benzoic acid had been transformed into hippuric acid (as in the case of the oat-fed rabbits), there would have been 0.0036 mol $\left(\frac{1.5}{122} \times \frac{50}{125} \times 0.73\right)$ of sodium hippurate in the 50 cc. of urine which were used for the analysis. Since 0.3 cc. of concentrated HCl is equivalent to 0.0036 mol $\left(\frac{0.3 \times 1.19 \times 0.37}{36.5}\right)$, just enough acid was used to convert the sodium hippurate in neutral aqueous solution into hippuric acid. Yet that same amount of acid was expected to be adequate for the conversion of the hippurate in an alkaline highly buffered urine into hippuric acid. In view of these facts, it is not surprising that Abderhalden and Wertheimer found such small quantities of hippuric acid in the alkaline urines of the rabbits on the green vegetable diet.

[3] Abderhalden and Wertheimer,[1] p. 461.

SUMMARY.

1. In these experiments the quantity of hippuric acid extracted by the method of Snapper and Laqueur (using 50 cc. of urine and 0.3 cc. of concentrated HCl) depended upon the reaction of the urine and not upon the actual amount of sodium hippurate present in the urine. This method was satisfactory only when less urine or more acid was used.

2. Although the character of the diet may regulate the synthesis of hippuric acid, the observations of Abderhalden and Wertheimer, based on faulty experimental data, cannot be considered as evidence for this regulation.

3. In our experiments, approximately normal formation of hippuric acid occurred in rabbits on a diet of green vegetables.

XUM

# A BIOCHEMICAL STUDY OF BONE GROWTH.

## I. CHANGES IN THE ASH, ORGANIC MATTER, AND WATER DURING GROWTH (MUS NORVEGICUS ALBINUS).

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This study was undertaken in order to make available a picture of the chemical differentiation of normal bone during growth. Such a picture serves two purposes. It affords a means of understanding the chemical development of a major system in vertebrates and hence enlarges the conception of the chemistry of development as a whole; and it provides a record wherewith the results of experimentation may be compared.

The analyses reported in this paper were made on the humerus and femur of male and female albino rats 23, 30, 50, 65, 75, 100, and 150 days of age. The results were derived from what may be called "standardized" material, and hence may themselves serve as "standards." Since the albino rat is more widely used than any other animal in studies of the effect of dietary and other disturbances on the skeleton the values are of particular interest in this connection.

The first two age groups (23 and 30 days) were each composed of ten pairs of males and ten pairs of females. Each pair was from a separate litter. There was thus available and used for analysis in these groups ten sets, of four bones of each kind to a set, from both sexes. It was necessary to use four bones combined in order to have sufficient ash for analysis. The age groups of 50, 65, 75, and 100 days were composed of ten animals of each sex, all from different litters. The 150 day old group contained 29 males and 28 females, also from separate litters. In these groups the analyses were made on the single bone. The methods of preparation and analysis have been given in an earlier paper (1). None of the rats were mated. They were all raised under

the same dietary and environmental conditions. The diet was composed of the varied mixture of foodstuffs described by Greenman and Duhring in their book on the "Breeding and care of the albino rat for research purposes" (2).

Table I gives the means of the results and their probable errors. The values for the 23 and 30 day groups are given in terms of the single bone.

TABLE I.—*Observed Mean Length, Weight, Ash, Organic Matter, and Water of the Humerus*

| Age. | Humerus. | | | | |
|---|---|---|---|---|---|
| | Length. | Weight. | Ash. | Organic matter. | Water. |
| | | | | | Male. |
| *days* | *mm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 23 | 13.4 ± 0.06 | 0.0557 ± 0.0020 | 0.0095 ± 0.0003 | 0.0100 ± 0.0004 | 0.0362 ± 0.0014 |
| 30 | 15.7 ± 0.08 | 0.0786 ± 0.0021 | 0.0139 ± 0.0006 | 0.0150 ± 0.0004 | 0.0497 ± 0.0012 |
| 50 | 18.3 ± 0.08 | 0.1018 ± 0.0016 | 0.0239 ± 0.0007 | 0.0203 ± 0.0006 | 0.0576 ± 0.0010 |
| 65 | 21.2 ± 0.15 | 0.1488 ± 0.0035 | 0.0472 ± 0.0018 | 0.0312 ± 0.0007 | 0.0705 ± 0.0014 |
| 75 | 21.9 ± 0.10 | 0.1591 ± 0.0028 | 0.0536 ± 0.0011 | 0.0330 ± 0.0005 | 0.0725 ± 0.0015 |
| 100 | 22.9 ± 0.21 | 0.1847 ± 0.0055 | 0.0703 ± 0.0028 | 0.0397 ± 0.0012 | 0.0747 ± 0.0016 |
| 150 | 25.9 ± 0.27 | 0.2556 ± 0.0041 | 0.1112 ± 0.0041 | 0.0572 ± 0.0018 | 0.0875 ± 0.0026 |
| | | | | | Female. |
| 23 | 14.0 ± 0.07 | 0.0573 ± 0.0017 | 0.0101 ± 0.0004 | 0.0111 ± 0.0003 | 0.0361 ± 0.0010 |
| 30 | 15.3 ± 0.06 | 0.0725 ± 0.0015 | 0.0131 ± 0.0004 | 0.0140 ± 0.0003 | 0.0454 ± 0.0008 |
| 50 | 18.2 ± 0.10 | 0.1008 ± 0.0019 | 0.0266 ± 0.0007 | 0.0206 ± 0.0005 | 0.0536 ± 0.0011 |
| 65 | 20.5 ± 0.13 | 0.1333 ± 0.0023 | 0.0423 ± 0.0016 | 0.0280 ± 0.0006 | 0.0631 ± 0.0008 |
| 75 | 21.2 ± 0.15 | 0.1417 ± 0.0037 | 0.0512 ± 0.0013 | 0.0296 ± 0.0008 | 0.0609 ± 0.0019 |
| 100 | 22.5 ± 0.15 | 0.1732 ± 0.0031 | 0.0729 ± 0.0019 | 0.0380 ± 0.0007 | 0.0623 ± 0.0011 |
| 150 | 24.0 ± 0.13 | 0.2033 ± 0.0050 | 0.0921 ± 0.0031 | 0.0464 ± 0.0011 | 0.0648 ± 0.0012 |

Direct examination of the basic data brings out certain significant relations.

Increase of bone weight with age is accomplished by a side by side increase in the amount of ash, organic matter, and water. A deviation from this orderly progression is exhibited by the water between the ages of 65 and 75 days. This element, instead of increasing, decreases in the bones of the female and remains constant in the femur of the male, during the period noted. It is evident that a disturbance of that phase of metabolism which is

represented by water increment with age occurs at this time, particularly in the female, even to the extent of a partial dehydration. It can be considered a reaction to a stimulus connected with the puberal phase of development which is about completed at this age. The phenomenon is quite in line with the observation of Hatai (3) that the orderly course of the curve of the refractive index of the blood serum of the rat on age is interrupted

*and Femur of Male and Female Albino Rats 23, 30, 50, 65, 75, 100, and 150 Days of Age.*

| Femur. | | | | |
|---|---|---|---|---|
| Length. | Weight. | Ash. | Organic matter. | Water. |
| *mm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 14.8 ± 0.09 | 0.0841 ± 0.0035 | 0.0112 ± 0.0005 | 0.0155 ± 0.0007 | 0.0575 ± 0.0024 |
| 18.4 ± 0.10 | 0.1373 ± 0.0041 | 0.0199 ± 0.0009 | 0.0263 ± 0.0009 | 0.0911 ± 0.0024 |
| 22.7 ± 0.13 | 0.2069 ± 0.0027 | 0.0417 ± 0.0014 | 0.0423 ± 0.0009 | 0.1229 ± 0.0018 |
| 27.0 ± 0.24 | 0.3255 ± 0.0085 | 0.0929 ± 0.0040 | 0.0705 ± 0.0016 | 0.1621 ± 0.0039 |
| 27.9 ± 0.22 | 0.3407 ± 0.0060 | 0.1073 ± 0.0022 | 0.0716 ± 0.0012 | **0.1619** ± 0.0034 |
| 29.7 ± 0.33 | 0.4050 ± 0.0131 | 0.1457 ± 0.0073 | 0.0884 ± 0.0034 | 0.1709 ± 0.0030 |
| 33.9 ± 0.33 | 0.5811 ± 0.0196 | 0.2417 ± 0.0110 | 0.1332 ± 0.0044 | 0.2062 ± 0.0064 |
| | | | | |
| 15.5 ± 0.10 | 0.0885 ± 0.0034 | 0.0128 ± 0.0006 | 0.0174 ± 0.0006 | 0.0584 ± 0.0022 |
| 17.9 ± 0.09 | 0.1258 ± 0.0031 | 0.0190 ± 0.0008 | 0.0246 ± 0.0007 | 0.0822 ± 0.0018 |
| 22.6 ± 0.16 | 0.2011 ± 0.0042 | 0.0472 ± 0.0013 | 0.0419 ± 0.0010 | 0.1121 ± 0.0028 |
| 26.1 ± 0.17 | 0.2866 ± 0.0042 | 0.0832 ± 0.0033 | 0.0611 ± 0.0013 | 0.1424 ± 0.0022 |
| 27.0 ± 0.21 | 0.3080 ± 0.0077 | 0.1042 ± 0.0029 | 0.0660 ± 0.0017 | **0.1378** ± 0.0017 |
| 29.0 ± 0.23 | 0.3818 ± 0.0090 | 0.1539 ± 0.0051 | 0.0849 ± 0.0021 | 0.1431 ± 0.0032 |
| 31.0 ± 0.30 | 0.4502 ± 0.0119 | 0.1944 ± 0.0071 | 0.1054 ± 0.0025 | 0.1505 ± 0.0036 |

at puberty. Data to be presented at a later stage of the analysis will demonstrate that the climax of the adjustment to sexual maturity affects the chemical differentiation of the bones and that loss of water is but one of the reactions induced thereby.

Age for age the bones of the female are smaller than those of the male save at 23 days. They also, with this exception, carry smaller amounts of organic matter and water. The situation at 23 days is correlatable with the fact that the female is a larger animal than the male at this time. This is seen from Table II

and is in agreement with Donaldson's records (4). While the ash values are consistent with the general relations at 23 days, at 50 and 100 days the bones of the female carry more ash than do those of the male, notwithstanding the fact that the male organism in general and the bones in particular are larger than those of the female, and carry more organic matter and water. This is a sex difference *sui generis* and indicates that the rate of ash deposition is greater in the female than in the male during the growth periods from 23 to 50 and from 75 to 100 days of age. The significance of this phenomenon will be discussed shortly.

TABLE II.

*Observed Mean Body Length and Body Weight of the Groups of Animals Used in This Study.*

| Age. | Male. | | Female. | |
|---|---|---|---|---|
| | Body length. | Body weight. | Body length. | Body weight. |
| *days* | *mm.* | *gm.* | *mm.* | *gm.* |
| 23 | 95.3 ± 0.7 | 27.3 ± 0.6 | 98.9 ± 0.8 | 28.5 ± 0.7 |
| 30 | 116.1 ± 0.8 | 41.3 ± 0.8 | 110.8 ± 0.6 | 38.7 ± 0.7 |
| 50 | 143.3 ± 0.6 | 74.5 ± 1.8 | 141.8 ± 1.0 | 74.2 ± 0.8 |
| 65 | 169.3 ± 1.2 | 120.8 ± 2.1 | 161.8 ± 0.9 | 105.0 ± 1.6 |
| 75 | 174.8 ± 1.2 | 133.2 ± 3.2 | 166.8 ± 1.3 | 115.8 ± 2.6 |
| 100 | 182.5 ± 1.6 | 162.3 ± 4.6 | 177.1 ± 0.9 | 137.6 ± 2.2 |
| 150 | 205.8 ± 1.6 | 244.3 ± 6.9 | 188.4 ± 1.3 | 174.7 ± 4.4 |

The percentage composition of the bones exhibits interesting relationships. The values given in Table III are based on the fresh weight.

The percentage of ash of the femur is less than that of the humerus in both sexes at all ages. Considered systemically the degree of ossification of the humerus is greater than that of the femur. It would be interesting to correlate this with the relative frequency and rate of repair of fractures of the two bones. A higher organic matter and water percentage is the compensation for the lower ash percentage of the femur. The differences in percentage composition of the two bones are more a matter of differences in ash and water percentage than of differences in percentage of organic matter. Nevertheless, there is a tendency for the bones to approach each other in value of percentage of ash with age, which

tendency is mirrored by a drawing apart with respect to the percentage of organic matter. The systemic interrelations in water percentage remain practically constant.

Both bones of the female have a higher percentage of ash at all ages than do those of the male. That is to say, the degree of ossification of the bones of the female is greater than that of the male. It would be interesting to correlate this with the relative incidence and rate of healing of fractures in the two sexes. While the systemic difference can be considered a structural matter, the sex difference is of more far reaching significance in view of the sex-specific need of the female for bone salts in reproduction. It is probable that the greater percentage of ash in the bones of the female is an expression of a preparation for this need.

Both bones of the female have a lower percentage of water at all ages than do those of the male. This reciprocal ash-water sex difference is the same in direction as the systemic difference noted earlier. It indicates that the water content of the bones is largely factored by the rate of ash deposition.

Both bones of the female have a higher percentage of organic matter at all ages than do those of the male. The grouping in sex difference is thus not the same as that in systemic difference. In the latter this correlation is absent. From what is known of the way in which bone is formed it is obvious that ash and organic matter percentage should exhibit like direction of difference when isotopic bones are being compared in the two sexes of the same species. It might also be expected that a similar association would be exhibited when serially homologous bones are compared. That such is not the case is evidence that structural differences may be determining factors of differences in bone composition. Furthermore, systemic divergency in ash percentage is greater in degree than sex divergency. This indicates that as far as this bone component is concerned isotopic relationship involves a greater compositional similarity in characterizing material than does systemic connection.

Age is also an important factor in the determination of the composition of the bones. The distribution of ash, organic matter, and water in the adult bone is quite different from that obtaining at puberty or weaning. A study of Table III brings out the direction of the age influence.

XUM

TABLE III.

*Mean Percentage of Ash, Organic Matter, and Water of the Humerus and Femur of Male and Female Albino Rats 23, 30, 50, 65, 75, 100, and 150 Days of Age.*

| Age. | Humerus. | | | Femur. | | |
|---|---|---|---|---|---|---|
| | Ash. | Organic matter. | Water. | Ash. | Organic matter. | Water. |
| | | | Male. | | | |
| *days* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 23 | 17.07 ± 0.37 | 17.97 ± 0.11 | 64.95 ± 0.40 | 13.32 ± 0.28 | 18.32 ± 0.18 | 68.36 ± 0.30 |
| 30 | 17.60 ± 0.41 | 19.06 ± 0.09 | 63.34 ± 0.45 | 14.37 ± 0.36 | 19.09 ± 0.14 | 66.54 ± 0.42 |
| 50 | 23.47 ± 0.54 | 19.88 ± 0.28 | 56.65 ± 0.74 | 20.12 ± 0.56 | 20.39 ± 0.22 | 59.49 ± 0.69 |
| 65 | 31.49 ± 0.62 | 20.97 ± 0.15 | 47.54 ± 0.67 | 28.33 ± 0.68 | 21.71 ± 0.25 | 49.96 ± 0.61 |
| 75 | 33.67 ± 0.37 | 20.76 ± 0.12 | 45.57 ± 0.44 | 31 52 ± 0.39 | 21.01 ± 0.09 | 47.47 ± 0.42 |
| 100 | 37.81 ± 0.39 | 21.49 ± 0.24 | 40.70 ± 0.42 | 35.58 ± 0.58 | 21.74 ± 0.14 | 42.68 ± 0.69 |
| 150 | 43.41 ± 0.65 | 22.19 ± 0.19 | 34.40 ± 0.67 | 41.32 ± 0.85 | 22.96 ± 0.18 | 35.72 ± 0.69 |
| | | | Female. | | | |
| 23 | 17.51 ± 0.25 | 19.37 ± 0.19 | 63.12 ± 0.28 | 14.32 ± 0.19 | 19.72 ± 0.23 | 65.96 ± 0.29 |
| 30 | 17.97 ± 0.33 | 19.29 ± 0.14 | 62.74 ± 0.42 | 15.02 ± 0.32 | 19.49 ± 0.16 | 65.50 ± 0.43 |
| 50 | 26.38 ± 0.49 | 20.46 ± 0.19 | 53.16 ± 0.47 | 23.51 ± 0.52 | 20.80 ± 0.18 | 55.69 ± 0.61 |
| 65 | 31.51 ± 0.76 | 20.93 ± 0.18 | 47.56 ± 0.86 | 28.84 ± 0.85 | 21.27 ± 0.20 | 49.89 ± 0.97 |
| 75 | 36.22 ± 0.41 | 20.92 ± 0.12 | 42.86 ± 0.43 | 33.81 ± 0.30 | 21.42 ± 0.11 | 44.77 ± 0.30 |
| 100 | 42.01 ± 0.53 | 21.92 ± 0.10 | 36.07 ± 0.60 | 40.12 ± 0.61 | 22.23 ± 0.17 | 37.65 ± 0.80 |
| 150 | 45.11 ± 0.51 | 22.85 ± 0.14 | 32.04 ± 0.50 | 42.98 ± 0.58 | 23.46 ± 0.23 | 33.56 ± 0.55 |

The percentage of water in both bones of both sexes decreases as the animals grow older. This in spite of the fact that the absolute amount of water increases with age. It is therefore clear that the increase in the water carried by the bones is less rapid than that of other constituents, and that the structures undergo a progressive dehydration as they grow older. This latter phenomenon has already been reported by Donaldson (4) and is an expression of a generalized reaction to age not particular to the osseous system, but in which the nervous system (5) and other organs participate (6).

The decrease in water percentage is compensated for by an increase in the percentage of ash and organic matter. This indicates that these elements are continuously displacing water from the bones. Increments in ash participate in this reaction to a preponderant degree for the increase in the percentage of this constituent is some 86 to 89 per cent of the decrease in water percentage. It is evident that organic matter increment is responsible only for from 14 to 11 per cent of the total dehydration.

The sex difference in degree of participation of ash deposition in dehydration is small but consistently greater than the systemic difference. This is what might be expected from the fact that the process of progressive dehydration with age is organically general.

If the percentage values be charted it is seen that the curves for ash and water simulate that of a monomolecular autocatalyzed reaction. A deviation from regularity obtains in both bones of the female at 65 days. The curves for organic matter are sex-specifically irregular, as can be told by inspection of Table III. A visualization of the deviations and their significance is best obtained by charting the rate of increment of the percentage values brought to a unit basis, at the various ages. Chart 1 gives the picture for the male and Chart 2, for the female.

It is evident that up to 75 days of age the percentage of ash increases somewhat more rapidly in the femur than in the humerus. It is this fact which accounts for the approximation of the two bones in their ash percentage with age, as noted earlier. Quantitatively the systemic differences in organic matter and water are too small, and the sex differences too irregular to make discussion worth while.

The percentage of ash in both bones of both sexes increases

XUM

more rapidly than the percentage of organic matter. This is a further demonstration of the fact that the displacement of water is largely a matter of ash deposition rather than a question of increment in organic matter. The difference is greatest during the growth period from 23 to 65 days of age. Apparently this is the period of marked differentiation, since from then on particularly from 100 days on, the percentage of ash and the percentage

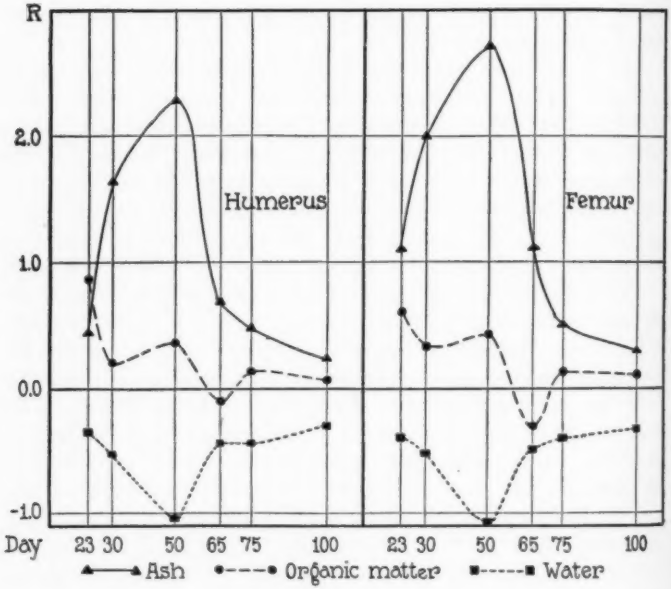


CHART 1. The rate of change in percentage of ash, organic matter, and water of the humerus and femur of the male.

of organic matter increase at practically the same low rate. Further analysis of this phase will be given presently.

Certain significant facts are brought out by a comparison of the qualitative relations of the curves on age.

The course of the age curves of the rate of increment in percentage for the three constituents is the same for both bones within each sex. This indicates a systemically uniform type of

response to the age factor in the chemical differentiation, at least as far as serially homologous bones are concerned.

The uniformity of the type reaction is, however, sex-specific, for the course of the curves in the male is quite different from that in the female. This indicates that sex factors play an important part in the determination of the course of chemical differentiation of the osseous system. Before going on to an analysis of the sex differences I will discuss the relations between the constituents.

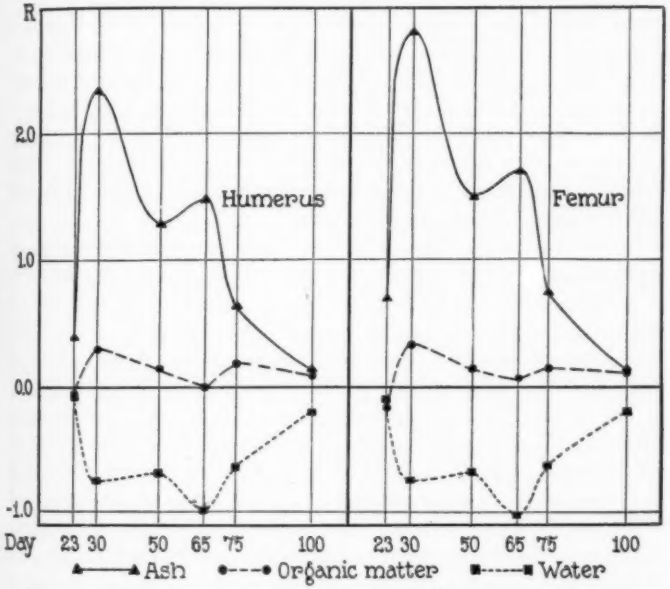


CHART 2. The rate of change in percentage of ash, organic matter, and water of the humerus and femur of the female.

The point of prime significance is the fact that the curve of the rate of increment in percentage of water is a reduced reflection of the curve for ash. That is to say the time of maximum increase in percentage of ash is the time of maximum decrease in percentage of water, and the time of minimum decrease in percentage of water is the time of minimum increase in percentage of ash. Intermediate points are similarly related. No such symmetry of relation obtains between organic matter and either water or ash.

It conclusively proves that the progressive deposition of ash during growth is the cause of the displacement of water, and that the increment in organic matter plays a relatively insignificant part in the dehydration which occurs with age. This of course has been inferred in an earlier portion of the analysis, but the demonstration of this parallelism throughout the growing period is the essential factor in the justification of the conclusion.

The course of the curve of the rate of increment in percentage of organic matter on age is the same for the humerus as for the femur in each sex. This systemic similarity is evidence that the changes have a meaning. What the meaning is, it is impossible to determine from these data. In the first place the changes are not consistently relatable to the changes in ash or water in a given sex. In the second place the direction of change with respect to ash and water differs in the two sexes up to the age of 75 days. This indicates that there is a marked sex difference in the chemical differentiation of bone up to puberty.

Up to 65 days the changes in rate of increment in percentage of organic matter differ in the two sexes. At 65 days a similar type of reaction occurs in both bones of both sexes. The increase in percentage of organic matter is practically stopped. This common reaction, however, does not have a common basis. In the male it is associated with an opposite change in water percentage; in the female with an opposite change in the ash percentage. Although the uniformity of type reaction is thus based on different adjustments in the two sexes, it is instructive as demonstrating the presence of a particular agency at this stage of development which causes a shift in bone composition equilibrium. The after reaction to the depression is in all cases an acceleration of the rate of increase in organic matter percentage which can be accounted for by the concomitant decrease in rate of increment in ash percentage. The subsequent change is in the direction of a diminution in rate of percentage of organic matter with which the water rather than the ash phase is associated. Thus it is evident that the shifts in organic matter percentage with age are not consistently relatable to shifts in either ash or water, and that interpretation is problematical. However, the fact that the course of the change in organic matter percentage and its relations to the other constituents after the depression at 65 days is the same for the

bones of both sexes, indicates that a common sex-indifferent type of differential growth has been arrived at.

Up to and including 65 days the age changes in the rate of increment in ash, water, and organic matter percentages are quite different in the male than in the female. The maximum rate of increment in ash percentage in the male occurs at 50 days of age. In the female the maximum is at 30 days. The female also shows a second maximum or acceleration at 65 days which is not exhibited by the male. The time of most rapid decrease in water percentage in the male precedes that in the female by 2 weeks. In the former it is at 50 days, in the latter at 65. Differences in organic matter percentage are also evident. These facts show that the course of chemical differentiation of the bones which produces the sex and systemically characteristic structure of the adult is determined by sex-specific influences.

The specificity of these influences is functioning in bone growth long before the allied influences determinative of the sex-characteristic functional alterations associated with puberty are superficially manifest.

The question arises as to whether or not these differences are correlated with differences in gonadal development. The introduction of the gonadal factor is justified on the basis of the belief that these organs are participants in the determination of sex-specificity in functional type. Now the histological studies of Allen (7) on the testis and Arai (8) on the ovary of the albino rat from the Wistar Colony show that these organs are undergoing puberal developmental changes. It is therefore clear that potentiality of prepuberal influence sex-specific in type emanating from these structures is present.

According to Allen (7) the first appearance of ripe spermatozoa occurs between the ages of 36 and 40 days at the time of the descent of the testes. This period coincides with the development of the maximum rate of increase in ash percentage of the bones in the male. According to Arai (8) the rapid decline in the number of ova in the ovaries stops during the period between 20 and 30 days of age. This coincides with the development of the first maximum rate of increase in percentage of ash in the bones of the female. At 64 days of age corpora lutea are first observed. This coincides with the acceleration in rate of increment in ash percentage in the bones of the female.

XUM

I believe that these sex-specific associations between the time of acceleration of ossification and definitive changes in gonadal development are indicative of a directing influence of the gonads on the chemical differentiation of bone. This belief is supported by the fact that the chemical differentiation of bone resulting in the composition characteristic for the mature animal is largely completed by the end of puberty (75 days) and that at that time and thereafter the rate of increment in percentage of ash, organic matter, and water shows sex differences of minor degree.

That this is directly concerned with sex differences in the metabolism of calcium is obvious. Earlier reports have noted a sex difference in calcium of the ash of the bones (9, 10), while Riddle and Honeywell (11) have recently noted a probable sex difference in blood calcium in pigeons. This phase of the analysis will be taken up in a succeeding paper where it will be shown that the percentage of calcium in the bones of the female is higher than in those of the male.

The systemic and sex differences in bone composition which have just been depicted are the result of the differences in rate of increment of the three constituents in the individual bones with age. A study of the changes in rate helps in an understanding of the chemical differentiation incident to growth for the individual bone, and in an understanding of the manner in which the systemic and sex differences in bone composition characteristic for the adult animal are arrived at.

In order to facilitate analysis the point of view of percentage rate, or "gm. per 100 gm. per day," has been taken instead of the usual conception of "gm. per day." By this procedure direct comparison on a common basis, and interpretation in terms of "growth capacity," or ability to add on increments in terms of initial unit quantity, is made possible. The utility of this method has been discussed in an earlier publication (12). Charts 3 and 4 give the growth capacity values of bone in weight, and the ash, organic matter, and water of the humerus and femur on age, of the male and female rats respectively.

The growth capacity of the femur in weight, and for ash, organic matter, and water is greater than that of the humerus in both sexes at all ages save one. At 65 days the humerus in the male

tends to show a higher capacity for growth in weight which is a consequence of the greater capacity for growth in organic matter and water, than does the femur. Although the absolute values are so small as to be almost negligible the change in relation is

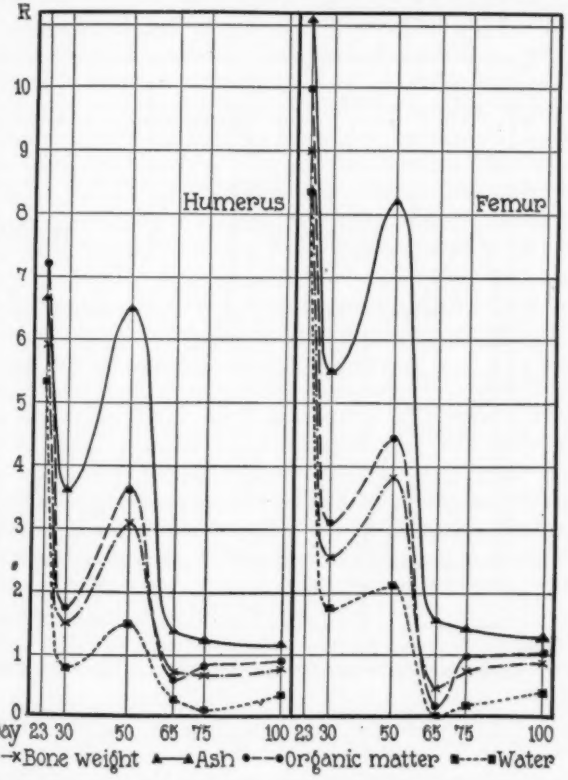


CHART 3. The growth capacity on age in bone weight, ash, organic matter, and water of the humerus and femur of the male.

probably real for this group of animals at least. An attempt at interpretation is hardly worth while until the matter has been confirmed. The general superiority of the femur indicates that this bone has an inherently greater growth impulse than its serial homologue.

In both sexes the direction of change in growth capacity with age for the three constituents is the same in the femur as in the humerus. This indicates that the influence of age upon the rate of growth of the bones in ash, organic matter, and water produces a response which is systemically typical.

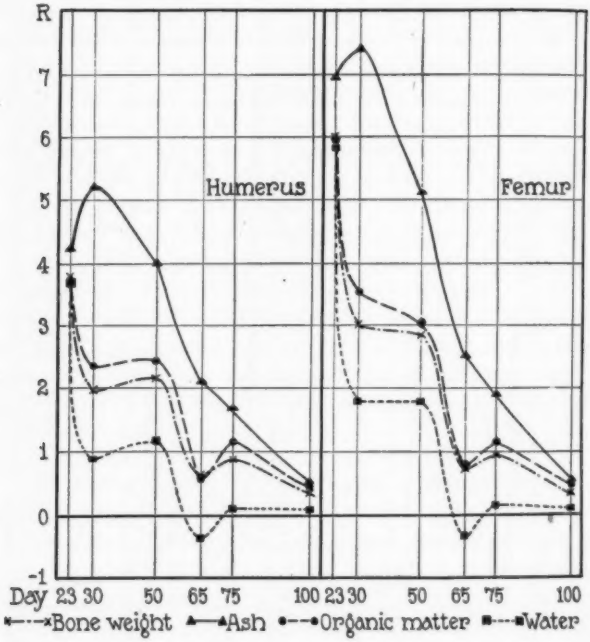


CHART 4. The growth capacity on age in bone weight, ash, organic matter, and water of the humerus and femur of the female.

In both sexes the values converge and the systemic differences in degree diminish so that at the attainment of sexual maturity (65 days) and from then on, the two bones differ but little in growth capacity in weight, or ash, organic matter, or water increments. Although the superiority of the femur is maintained, the difference is very small. The significance of this approximation will be developed shortly.

Turning now to the sex comparison, it is seen that there is a marked sex similarity in degree and type of change in growth capacity in water with age, a marked sex dissimilarity in growth capacity in ash, while the growth capacity in organic matter shows a sex-specificity greater than that of water, but less than that of ash. These differences are most evident during the growth period from 23 to 65 days of age. A tendency to convergency of the values leads to a general diminution of the sex differences so that at 65 days and thereafter there is but little actual dissimilarity.

In general the changes in direction of the course of the growth capacity values with age are the same for all constituents in the bones of the male. This does not obtain in the female. While organic matter and water tend to run similar courses, the rate of ash deposition follows an apparently independent path. This is shown by the fact that there occurs in the bones of this sex an acceleration of rate in ash increment at 30 days which is not exhibited by either organic matter or water. Other details are evident in the charts, but further elaboration is unnecessary.

This indicates that the relation of ash deposition to bone growth by increments in organic matter and water is different in the male than in the female, and that while in the one sex there is an apparent uniformity in type of growth impulse with age, in the other some factor exerts an influence upon growth by ossification independently of the general stimulus to growth by increments of organic matter and water. That is to say there is a sex-specific apparently independent factor for ossification in the female which is not exhibited in the male.

The age influence on rate of ash deposition in the female also differs from that in the male in the character of the changes produced at stated periods. The acceleration at 30 days does not obtain in the male. Instead a profound retardation is produced. This retardation is followed by an acceleration at 50 days, while in the female the gradual decrease in rate of growth by increments of ash has begun. From 50 days on the growth capacity for ash of the bones of the female falls away rather regularly to the end of the period of observation. In the male, on the other hand, an abrupt decrease occurs between 50 and 65 days of age, and thereafter but little alteration in rate occurs. This difference in the prepuberal tendency of the acceleration of rate of ash deposition is real. The interpretation has been given under a previous heading.

Apparently the bones of the male reach a stage of ossification rate at 65 days of age, coincident with the completion of sexual maturity, which is characteristic of stabilized adult growth, while in the female this adjustment is not completed until the later age of 100 days. This carrying past puberty of a relatively high rate of ossification by the female may be an expression of a preparation for the sex-specific needs of reproduction. This hypothesis is strengthened by the observation that the optimum reproduction capacity of the albino rat is not attained until the female is about 120 days old.

The sex-similarity in the growth capacity values for water as contrasted with the sex-dissimilarity in organic matter and ash shows that growth by increments in water is more independent of the sex-conditioned factors concerned in the production of sex-characteristic bone than is growth by increments in the other two constituents.

The interconstituental comparison shows that in both bones of both sexes the growth capacity for ash is greater than that for organic matter, water, or bone as a whole. This might be expected from the fact that the deposition of ash substance is the process by which bone is characterized. In both bones of both sexes the growth capacity for water is less than that of the other constituents and the bone as a whole. In the female at 65 days of age a negative value is recorded due to an actual loss of water at this time. The relative rates of ash and water increments with respect to the growth capacity of the bone in total weight are consistent with the conclusion made in an earlier paragraph that the decrease in water percentage is largely a matter of ash deposition.

The values of the growth capacity for organic matter in both bones of both sexes are strikingly like those of the bone as a whole, which is less than those for ash and greater than those for water. The almost exact identity in the values for the two rates may be taken as an expression of the fact that the rate of bone growth in weight is fundamentally conditioned by the rate of growth and activity of the organic matter (representative of protoplasmic activity in bone formation), and that the participation of the rate of ash deposition and water increment in the determination of the bone weight are dependent on this factor.

It is to be noted that, with the exception of the ash in the bones

of the female, there occurs a marked drop in growth capacity of all constituents and the bone as a whole at 30 days. Apparently some factor comes into play after 23 days of age which results in the retardation of bone growth. This factor probably arises from the adjustment to the new feeding conditions incident to weaning. A similar disturbance in the refractive index of the blood serum at this time has been recorded by Hatai (3). The evidence is clear that weaning produces a profound change in the metabolic activities of the organism and that these changes are reflected not only in the composition of the blood but also in the chemical differentiation of the osseous system.

There is a second abrupt and general decrease in growth capacity at 65 days of age. Since it is between the ages of 50 and 65 days that the albino rat is experiencing the acute stages of puberal development the conclusion is justified that the retardation is due to this physiological state. The retardation in growth capacity differs in degree for the various constituents in such a manner that the values tend to approach a common low level of rate in increment characteristic of adult growth at which the absolute differences exhibited are negligibly small as compared with preceding stages. This approximation of the hitherto diverse values occurs not only in the interconstituental, but also in the sex and systemic relations. It is not only an approximation, it is also a stabilization, for from 65 days on, the changes in the growth capacities are slight in comparison with what have gone before.

The approximation of the growth capacity values and general stabilization of growth rate occurs sharply at 65 days in the male and is complete by 75 days. In the female the adjustment, while marked at 65 days, is not complete until the rats are 100 days old. The delay in equilibration is probably an expression of a preparation on the part of the female for the sex-specific need for bone salts in reproduction.

At 23 days of age the growth capacity for ash, water, organic matter, and the bone as a whole fall closely together. They separate widely at 30 and 50 days, maintaining sex and systemically characteristic relations. At 65 days they again approach each other closely and from then on tend to become all of the same order of magnitude. This indicates that the growth period from 23 days of age through puberty is a period of constituental differ-

entiation, which differentiation is sex-characteristic in type and systemically characteristic in degree. The climax of puberty at 65 days of age abruptly brings to an end these major differences. It is thus evident that the growth factors productive of the bone composition characteristic of the individual structure as a part of the osseous system and of the sex exert their determining influence prior to attainment of sexual maturity, and that the completion of puberal development is the deciding factor in the adjustment of the growth capacity to a common stabilized level.

It is important to note that sex differences in constituental make-up and growth capacity values exist at 23 days of age. It is evident that these have been previously determined, probably at the time of sex determination. Since the sex-characteristic percentage composition of the bones is the same in the adult animal as in the nursling it is apparent that this is a relation determined at the time sex is determined. The sex differences in course of development of the bones subsequent to 23 days of age, while obviously ultimately determined when sex is determined, are, however, immediately determined by the sex-specific factors related to puberal development and hence are distinguishable in their origin from the differences which are present before their inception and the constant differences in percentage composition.

This analysis would be incomplete were attention not directed to the differential development with respect to bone growth in length. The data in Table I allow a computation of the amounts of ash, organic matter, and water per unit bone length at the different ages. A brief discussion of the relations can be made without taking the space necessary for their tabulation.

Both bones of both sexes increase to a greater degree in weight than in length during the period of observation. This superiority is due to the fact that the amount of ash and organic matter per unit length of bone increases continuously from 23 to 150 days of age. The increase in ash is the greater determinant and becomes increasingly so after 30 days of age. The increase in the amount of water per unit length of bone is of negligible importance in the case of the humerus, while in the femur it plays a rôle, though a minor one, up to the age of 65 days. Thereafter, even in this bone, the greater weight increase is due to ash and organic matter. This practical constancy of the water content of the humerus

after 30 and the femur after 65 days of age, per unit length of bone, in its relation to the changes in ash or organic matter is significant, in its contrast with the interconstituental relations on the weight basis, of the conditioning effect of structural development on bone composition. This is emphasized by the systemic difference in the change in water content per unit length on age, and by the fact that the amount of ash, organic matter, and water per unit length of the femur is greater than that of the humerus in both sexes at all ages. This latter is a chemical demonstration that the humerus is more slender than the femur. The amount of water per unit length of both bones is consistently greater in the male than in the female. The sex differences in organic matter are inconsequential and the differences in ash are inconsistent. These relations taken with the fact that the bones of the female are more slender than those of the male are merely a further demonstration that the degree of ossification per unit length is greater in the former than in the latter sex.

## SUMMARY AND CONCLUSION.

An analysis has been made of the systemic, sex, and constituental differences in composition and differentiation with age of the humerus and femur of male and female albino rats. The analysis was based on the values obtained for ash, organic matter, and water contents, together with the fresh weights and lengths of the bones.

The more significant findings are as follows:

1. The ash percentage of the humerus is greater than that of the femur in both sexes at all ages. This signifies that the degree of ossification of the humerus is greater than that of the femur (Table III).

2. Both bones in the female have a higher percentage of ash than do those of the male. This indicates that the degree of ossification of the bones of the female is greater than that of the male (Table III).

3. The percentage of water of both bones of both sexes decreases with age. This is largely due to ash deposition. The increase in percentage of organic matter is a minor factor (Table III).

4. The sex-specific differences in the course of differential development can be traced to gonadal influences.

XUM

5. A marked drop in the growth capacity occurs at 30 days of age. This is attributed to the adjustment to the new feeding conditions following weaning at 23 to 25 days of age (Charts 3 and 4).

6. There is a second abrupt and general decrease in growth capacity at 65 days of age. This is attributable to the adjustment to puberty (Charts 3 and 4).

7. Evidence is had that the growth period from 23 days of age through puberty is a period of constituental differential development, which is sex-characteristic in type and systemically characteristic in degree. The climax of puberty at 65 days of age abruptly brings these major differences to an end (Charts 3 and 4).

Grateful acknowledgment should be made to Mr. H. L. Hinski and to Miss Elizabeth Justice for their participation in the analytical work.

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# THE CALCIUM CONTENT OF THE BODY IN RELATION TO AGE, GROWTH, AND FOOD.*

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Fundamental to the complete interpretation of the data of many recent investigations upon the relation of food to growth and to the development of bone, is a more quantitative knowledge than has hitherto existed as to the normal calcium content of the body at different stages of its life history.

Both for our understanding of the rôle of calcium in nutrition and to enable us fully to appreciate the significance of calcium as a factor in food values, it is important to know, as accurately as we may, the endowment of calcium received by the young mammal from its mother before birth and during the suckling period, the rate at which calcium must be stored during growth in order to provide for the normal development of the body, how much and how long the relation of body calcium to body weight normally changes with growth and development, whether age continues to be a factor after growth and development have been completed, and whether the bearing and suckling of young normally reduces the store of calcium which the female has acquired during her growth and development.

The present paper records the results of a somewhat extended series of quantitative determinations of calcium in normal animals of both sexes at different ages, including separate groups of females which had and had not reared young, and a series of studies of the influence of several variations in the food supply upon the calcium content of the body. The rat, as an omnivorous mammal whose general development has been well studied in connection with many other investigations and which is being widely and increas-

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ingly used in nutrition research and in testing food values, was chosen as the experimental animal for this work.

While our analyses were in progress, Buckner and Peter published (1) a series of determinations of phosphorus, calcium, magnesium, and potassium in rats of from 2 to 40 weeks of age; but concluded that their data were not sufficient for the establishment of normal standards inasmuch as "individual variation was not overcome" in their averages of three animals for a given age and sex.

As Buckner and Peter describe their system of feeding, it would appear to the present writers that the irregular results obtained in the analyses of their rats may have been due not wholly to individual differences as that expression is usually understood, *i.e.* differences appearing without otherwise accountable cause, but also, in part at least, to the fact that the diet of their experimental animals was not (in our judgment) sufficiently controlled. They stated regarding the animals analyzed by them:

> "After weaning, their principal food consisted of cracked yellow corn which was kept before them at all times. Three times a week stale light bread soaked in fresh whole milk was fed as the morning meal in such quantities as they would consume in 30 minutes and twice a week shredded cabbage was fed. Laboratory tap water was used throughout. They were bedded with clean oat straw which was changed twice a week."

Thus their animals, although all offered the same food supply, may have actually consumed the corn, the bread and milk, and the cabbage in different quantitative proportions; and since the different foods, thus offered unmixed and not in individual weighed portions to each rat, are quite different in calcium content, it must follow that variations in individual preferences for one or another of these foods or in the palatability of either of the fresh foods as supplied to different lots of rats at different times, might cause very material differences in both the absolute and relative calcium intakes of the animals. The use of oat straw for bedding introduces another source of uncertainty, for while the amount of this consumed by the animals was probably small it was probably also variable. From our experience with different diets we are led to believe that the food supply of the rats described by Buckner and Peter was probably below the optimum in calcium content, and in general the growth and development of animals on such

diets is more variable than that obtained on diets which are richer in their supplies of the needed mineral elements.

The diet of the rats which we have analyzed as normals was richer in calcium than we judge that of Buckner and Peter to have been, and was prepared from solid foods so thoroughly ground and mixed together that all of the rats recorded as living on a given diet must actually have eaten a food mixture of exactly the same composition. Our rats were kept in all-metal cages without bedding and with constant access to the food mixture and to distilled water.

*Calcium Contents of Normal Rats at Different Ages.*

The rats used for this study of the normal calcium content of the body at different ages were raised on a diet consisting essentially of a dry food mixture of which one-third was whole milk powder and two-thirds was ground whole wheat, or in a few cases white flour. In some cases definitely known limited amounts of fresh meat or fresh vegetable were fed in addition, which in the quantities here fed were found not to influence the results so far as concerns the calcium content of the body. A few of the rats analyzed (indicated by an asterisk (*) in the tables) had received a larger proportion of milk powder; *viz.*, one-half of the dry food mixture. This, although somewhat increasing the calcium intake, did not prove to have any appreciable effect upon the calcium content of the animals.

Inasmuch as these modifications of our most used diet (Diet B; Diet 13), rendering it richer in one or more of the factors, protein, vitamins, and calcium, did not increase the calcium content of the body, and furthermore since this same Diet B has served in this laboratory for the normal nutrition of fifteen successive generations without the use of any other food whatever, it seems adequately established that this diet may be taken as supporting normal nutrition from whatever point of view regarded, and therefore that rats raised on this diet, or any modification of it shown to yield equivalent results, may properly be taken as suitable material for analysis for the purpose of establishing the normal calcium content of the body at different ages.

Such animals were taken for analysis at birth, 15, 30, 60, 90, and 120 days. After the age of 120 days, animals were not

analyzed at such definite intervals, but sufficient data have been determined to show the trend of the curves through the early adult lives of both sexes. In the cases of the animals taken for analysis at birth, sexes were not determined and in one case the young of a litter were analyzed together. Beginning at the age of 15 days, however, the sexes were always determined and each animal taken for analysis was analyzed separately.

*Procedure.*

The animals were killed by chloroform, the alimentary tracts removed (except in the cases of the new-born animals), and the bodies then ashed in silica dishes. Other work in this laboratory has shown that of the total calcium in the rat's body about 99 per cent is in the skeleton and that the amount contained in the walls of the alimentary tract is entirely negligible so that the rejection of the walls of the tract can have no appreciable effect upon the determination of the total calcium content of the body. In order to obtain the true body weight of the animal, the contents of the alimentary tract were removed and weighed and this weight was subtracted from the live weight.

The entire rat minus its alimentary tract was first charred slowly over a small flame and then ashed at a low temperature in the muffle. The ash was dissolved in hydrochloric acid, the solution filtered and made up to such a volume that 25 cc. of the solution would contain about 0.05 to 0.1 gm. of calcium. The calcium was determined by the McCrudden gravimetric method (2). 25 cc. of the solution were measured and diluted to 75 cc. with distilled water. This solution was made just alkaline, using 2.5 M ammonium hydroxide, and then just acid with molar hydrochloric acid in the presence of azolitmin as indicator. 3.5 cc. of molar hydrochloric acid and 10 cc. of 2.5 per cent (0.20 M) oxalic acid were then added. The solution was boiled and an excess of 3 per cent (0.21 M) ammonium oxalate was added to the boiling solution. When the mixture was cold, 10 cc. of 16 per cent (1.18 M) sodium acetate were added. The precipitate of calcium oxalate was allowed to stand overnight at room temperature. The precipitate was filtered on No. 44 Whatman ashless filter paper, washed with 0.5 per cent (0.04 M) ammonium oxalate solution until free from chloride ion, ignited, and weighed as calcium oxide.

RESULTS AND DISCUSSION.

The results are shown in Tables I to IX and Figs. 1 to 4. It will be seen that the average calcium content here found in the

TABLE I.

*Calcium Content of New-Born Rats.*

| Rat No. | Body weight. | Calcium in body. | |
|---|---|---|---|
| | *gm.* | *gm.* | *per cent* |
| 8 young of No. 11795.* | 4.5 | 0.0118 | 0.26 |
| 21 | 3.4 | 0.0075 | 0.22 |
| 22 | 4.1 | 0.0080 | 0.20 |
| 56 | 5.3 | 0.0109 | 0.21 |
| 57 | 5.6 | 0.0112 | 0.20 |
| 58 | 5.3 | 0.0116 | 0.22 |
| 102 | 5.5 | 0.0130 | 0.24 |
| 120 | 4.0 | 0.0126 | 0.31 |
| Average | 4.7 | 0.0108 | 0.25 |

* This line gives average data for eight young of No. 11795 which were chloroformed when less than 24 hours old and analyzed together for calcium.

TABLE II.

*Calcium Content of 15 Day Old Rats.*

| Rat No. | Body weight. | Calcium in body. | |
|---|---|---|---|
| Males. | | | |
| | *gm.* | *gm.* | *per cent* |
| Young of No. 7901. | 24 | 0.1412 | 0.59 |
| " " " 7901. | 24 | 0.1368 | 0.57 |
| " " " 8310. | 22 | 0.1298 | 0.59 |
| " " " 8310. | 22 | 0.1275 | 0.58 |
| " " " 8310. | 23 | 0.1310 | 0.57 |
| Average | 23 | 0.1333 | 0.58 |
| Females. | | | |
| Young of No. 7901. | 24 | 0.1431 | 0.60 |
| " " " 7901. | 23.5 | 0.1384 | 0.59 |
| " " " 7901. | 23.5 | 0.1388 | 0.59 |
| " " " 7901. | 23 | 0.1324 | 0.58 |
| " " " 7901. | 22.5 | 0.1361 | 0.61 |
| " " " 7803. | 22 | 0.1375 | 0.63 |
| Average | 23 | 0.1377 | 0.60 |

new-born rat was 0.0108 gm. or 0.25 per cent. A rapid increase in percentage of calcium in the rat was found to take place during the first 15 days of life. While the weight of the rat increases about 5-fold during this period, the weight of calcium increases about 12-fold rising to an average of 0.58 per cent in males and 0.60 per cent in females. The weights and percentages of calcium in the body also show marked successive increases at 30, 60, and

TABLE III.

*Calcium Content of 30 Day Old Rats.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| | | Males. | | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 476 | 28 | 45 | 45 | 0.315 | 0.71 |
| 561 | 30 | 57 | 56 | 0.384 | 0.69 |
| 721 | 29 | 41 | 39 | 0.271 | 0.69 |
| 103 | 27 | 31 | | 0.214 | 0.69 |
| 2410 | 30 | 63 | 61 | 0.420 | 0.69 |
| 8027 | 28 | 40 | 39 | 0.263 | 0.67 |
| Average... | 29 | 46 | 45 | 0.311 | 0.69 |
| | | Females. | | | |
| 465 | 34 | 29 | 29 | 0.245 | 0.79 |
| 566 | 30 | 43 | 42 | 0.295 | 0.70 |
| 726 | 29 | 41 | 40 | 0.292 | 0.74 |
| 815 | 30 | 47 | 46 | 0.373 | 0.81 |
| 167 | 33 | 46 | | 0.326 | 0.71 |
| 2247 | 30 | 49 | 49 | 0.345 | 0.71 |
| Average... | 31 | 43 | 42 | 0.313 | 0.74 |

90 days. At 30 days the average percentages here found for males and females were, respectively, 0.69 and 0.74 per cent; at 60 days, 0.76 and 0.86 per cent; at 90 days, 0.93 and 1.09 per cent. At 120 days (Table VI) it becomes apparent that rate of increase of calcium is less rapid; but the data indicate that the percentage of calcium in the bodies of males and of females which have not raised young increases slowly up to about the age of 1 year, after which it remains fairly constant.

The very rapid increase in body calcium which takes place in the rat during normal growth and development is perhaps most clearly seen from a comparison of the gain in body weight with the gain in weight of calcium in the body, as shown in Fig. 4. Here the average data of our observations on normal male albino rats

TABLE IV.

*Calcium Content of 60 Day Old Rats.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| | | Males. | | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 430 | 60 | 149 | 144 | 1.122 | 0.78 |
| 431 | 60 | 120 | 115 | 0.891 | 0.77 |
| 613 | 61 | 160 | 158 | 1.182 | 0.75 |
| 642 | 62 | 123 | 120 | 0.931 | 0.78 |
| 616 | 61 | 121 | 118 | 0.839 | 0.71 |
| 583 | 60 | 127 | 123 | 1.003 | 0.81 |
| 2468 | 60 | 105 | 100 | 0.801 | 0.80 |
| 1521 | 60 | 120 | 117 | 0.862 | 0.74 |
| 9228 | 61 | 144 | 139 | 1.042 | 0.75 |
| 9223 | 61 | 170 | 165 | 1.259 | 0.76 |
| 9157 | 60 | 184 | 176 | 1.316 | 0.75 |
| Average... | 61 | 138 | 135 | 1.023 | 0.76 |
| | | Females. | | | |
| 436 | 60 | 104 | 100 | 0.829 | 0.83 |
| 646 | 62 | 111 | 107 | 0.889 | 0.83 |
| 758 | 60 | 120 | 115 | 0.958 | 0.83 |
| 1522 | 60 | 93 | 90 | 0.775 | 0.86 |
| 1523 | 60 | 94 | 91 | 0.765 | 0.84 |
| 1566 | 60 | 142 | 138 | 1.203 | 0.86 |
| 2472 | 60 | 88 | 84 | 0.779 | 0.93 |
| 9162 | 60 | 133 | 128 | 1.110 | 0.87 |
| Average... | 60 | 111 | 107 | 0.914 | 0.86 |

are plotted with reference to age and it will be seen that in reaching adult size and development these rats multiplied their birth weights by about 70 and the weights of calcium contained in their bodies at birth by about 360.

At all ages the percentage of calcium in females which had not

raised young was higher than that in males of the same age which had received the same diet. There is an indication of this difference at 15 days, the males averaging 0.58 per cent and the females

TABLE V.

*Calcium Content of 90 Day Old Rats.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| | | | Males. | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 567 | 90 | 208 | 203 | 1.872 | 0.92 |
| 500 | 90 | 244 | 237 | 2.374 | 1.00 |
| 503 | 90 | 225 | 220 | 2.015 | 0.92 |
| 504 | 90 | 213 | 207 | 2.024 | 0.98 |
| 651 | 91 | 235 | 231 | 2.173 | 0.94 |
| 650 | 91 | 258 | 253 | 2.255 | 0.89 |
| 1138 | 94 | 183 | 183 | 1.789 | 0.98 |
| 1216 | 87 | 217 | 212 | 1.991 | 0.94 |
| 1606 | 90 | 239 | 233 | 2.127 | 0.91 |
| 1607 | 90 | 240 | 235 | 1.966 | 0.84 |
| 2444 | 89 | 156 | 149 | 1.544 | 1.04 |
| 9172 | 90 | 236 | 229 | 2.026 | 0.89 |
| 9163 | 90 | 233 | 226 | 2.014 | 0.89 |
| Average... | 90 | 222 | 217 | 2.013 | 0.93 |
| | | | Females. | | |
| 9232 | 90 | 150 | 146 | 1.622 | 1.11 |
| 505 | 90 | 165 | 160 | 1.729 | 1.08 |
| 506 | 90 | 193 | 189 | 1.990 | 1.05 |
| 507 | 90 | 168 | 163 | 1.826 | 1.12 |
| 1141 | 94 | 128 | 126 | 1.348 | 1.07 |
| 1218 | 87 | 148 | 144 | 1.511 | 1.05 |
| 1668 | 90 | 169 | 166 | 1.885 | 1.14 |
| 1719 | 90 | 160 | 157 | 1.682 | 1.07 |
| 2447 | 89 | 147 | 141 | 1.625 | 1.15 |
| 11171 | 90 | 176 | 171 | 1.822 | 1.07 |
| 11172 | 90 | 169 | 165 | 1.794 | 1.09 |
| Average... | 90 | 161 | 157 | 1.712 | 1.09 |

0.60 per cent. At 30, 60, and 90 days there is no doubt that the difference between the sexes is significant, as also at later ages (Tables VI, VII, and VIII).

TABLE VI.

*Calcium Content of 120 Day Old Rats.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| | | | Males. | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 527 | 116 | 268 | 263 | 2.573 | 0.98 |
| 580 | 123 | 242 | 236 | 2.316 | 0.98 |
| 1059 | 116 | 245 | 240 | 2.391 | 1.00 |
| 1133 | 115 | 227 | 222 | 2.288 | 1.03 |
| 1135 | 115 | 215 | 211 | 2.233 | 1.06 |
| 1329 | 120 | 280 | 270 | 2.662 | 0.99 |
| 1464 | 122 | 253 | 244 | 2.475 | 1.02* |
| 1053 | 115 | 332 | 327 | 3.210 | 0.98* |
| Average... | 118 | 258 | 252 | 2.517 | 1.01 |
| | | | Females which had borne no young. | | |
| 528 | 116 | 182 | 175 | 1.914 | 1.09 |
| 581 | 123 | 195 | 190 | 1.953 | 1.03 |
| 7158 | 126 | 174 | 167 | 1.661 | 1.00 |
| 5685 | 120 | 211 | 204 | 2.250 | 1.10 |
| 5686 | 120 | 190 | 184 | 2.098 | 1.14 |
| 5687 | 120 | 190 | 184 | 2.135 | 1.16 |
| 588 | 121 | 217 | 213 | 1.993 | 0.94†‡ |
| 589 | 121 | 186 | 183 | 1.983 | 1.08‡ |
| Average... | 121 | 193 | 188 | 1.998 | 1.07 |
| | | | Females which raised one litter. | | |
| 1277 | 122 | 205 | 201 | 1.884 | 0.94 |
| 1285 | 126 | 170 | 164 | 1.641 | 1.00 |
| 1284 | 130 | 185 | 180 | 1.780 | 0.99 |
| Average... | 126 | 187 | 182 | 1.768 | 0.98 |

* These were animals from a slightly different diet as explained in the text.

† No. 588 weighed 190 gm. 1 week before it was killed. When the percentage is calculated on this weight it becomes 1.05. If this figure be used, the average is changed to 1.08.

‡These two were pregnant; others had not bred at all.

The percentage of body calcium in females which have raised young is lower than in those which have not raised young. For the cases here studied it was also lower than in males of the same age which had been raised on the same diet.

TABLE VII.

*Calcium Content of Adult Male Rats.*

| Rat No. | Age. | Last live weight. | Net weight. | Maximum weight. | Calcium in body. | |
|---|---|---|---|---|---|---|
| | *days* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent* |
| 1239 | 179 | 282 | 276 | 282 | 2.819 | 1.02* |
| 1054 | 185 | 322 | 313 | 326 | 3.412 | 1.09* |
| 1055 | 185 | 323 | 316 | 323 | 3.167 | 1.00* |
| 1063 | 185 | 271 | 267 | 279 | 2.763 | 1.04 |
| 1064 | 185 | 260 | 255 | 264 | 2.697 | 1.06 |
| 259 | 206 | 367 | 359 | 367 | 3.914 | 1.09 |
| Average.. | 188 | 304 | 298 | 307 | 3.132 | 1.05 |
| 627 | 245 | 365 | 359 | 383 | 3.876 | 1.01† |
| 628 | 245 | 344 | 336 | 350 | 3.708 | 1.06† |
| 6118 | 245 | 244 | 238 | 257 | 2.684 | 1.04† |
| 6116 | 245 | 303 | 297 | 312 | 3.185 | 1.02† |
| 667 | 241 | 342 | 334 | 355 | 3.831 | 1.08† |
| 816 | 241 | 346 | 340 | 359 | 4.034 | 1.12† |
| 817 | 241 | 347 | 341 | 364 | 4.012 | 1.10*† |
| 818 | 241 | 371 | 363 | 375 | 4.087 | 1.09*† |
| 1573 | 245 | 313 | 305 | 313 | 3.134 | 1.03*† |
| Average.. | 243 | 331 | 324 | 341 | 3.617 | 1.06 |
| 164 | 309 | 330 | 325 | 338 | 3.891 | 1.15† |
| 666 | 416 | 358 | 351 | 371 | 4.066 | 1.10† |
| 40 | 485 | 365 | 358 | 383 | 4.122 | 1.08† |
| 570 | 522 | 313 | 305 | 317 | 3.274 | 1.07 |
| 740 | 603 | 292 | 285 | 350 | 4.040 | 1.15† |
| 7 | 849 | 339 | 334 | 395 | 4.068 | 1.03 |

*These were animals from a slightly different diet as explained in the text.

†Percentage calculated on basis of maximum weight.

The youngest of our age groups which contained females which had raised young was that for rats 120 days old. Beginning with 120 days, therefore, we have, in charting our results (Figs. 1 and 2) given two curves for the calcium content of females according as

they have or have not raised young (Tables V to VIII and Figs. 1 and 2).

We have also obtained a limited amount of evidence bearing upon the question as to whether and at what rate a female which has raised young recovers the calcium lost in the process.

TABLE VIII.

*Calcium Content of Adult Female Rats Which Had Reared No Young.*

| Rat No. | Age. | Last live weight. | Net weight. | Maximum weight. | Calcium in body. | |
|---|---|---|---|---|---|---|
| | *days* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent* |
| 4670 | 179 | 231 | 227 | 231 | 2.378 | 1.05 |
| 4765 | 190 | 178 | 175 | 193 | 2.056 | 1.07* |
| 4767 | 190 | 160 | 156 | 164 | 1.836 | 1.18 |
| 4729 | 198 | 244 | 240 | 244 | 2.815 | 1.17 |
| Average.. | 189 | 203 | 200 | 208 | 2.271 | 1.12 |
| 6873 | 182 | 201 | 195 | 210 | 1.812 | 0.93† |
| 319 | 181 | 197 | 192 | 202 | 2.273 | 1.18‡ |
| 247 | 227 | 220 | 215 | 220 | 2.548 | 1.19 |
| 820 | 240 | 244 | 239 | 244 | 2.629 | 1.10 |
| 3909 | 242 | 221 | 218 | 224 | 2.716 | 1.25 |
| 631 | 245 | 242 | 237 | 251 | 2.945 | 1.17* |
| 1579 | 245 | 220 | 217 | 224 | 2.550 | 1.18§ |
| 215 | 244 | 214 | 212 | 214 | 2.630 | 1.26 |
| Average.. | 243 | 228 | 225 | 231 | 2.694 | 1.19 |
| 821 | 264 | 229 | 224 | 229 | 2.767 | 1.24§ |
| 823 | 264 | 210 | 205 | 210 | 2.671 | 1.30§ |
| 196 | 269 | 222 | 218 | 222 | 2.801 | 1.29 |
| Average.. | 266 | 220 | 216 | 220 | 2.746 | 1.28 |

* Percentage calculated on basis of maximum weight.

† No. 6873 had six young separated from her on the day she was killed.

‡ No. 319 had two young separated from her 2 weeks before she was killed.

§ These were animals from a slightly different diet as explained in the text.

Five 8 month old females which had raised no young showed an average of 2.694 gm. of calcium or 1.19 per cent. Seven females of the same age which had raised young showed an average of 2.267 gm. of calcium or 1.05 per cent. Here the bearing and suckling

of young had on the average evidently reduced the female's body calcium by from 10 to 16 per cent whether considered from the standpoint of the absolute weight or the percentage of calcium in the body.

TABLE IX.

*Calcium Content of Adult Female Rats Which Had Reared Young.*

| Rat No. | Age. | Last live weight. | Net weight. | Maximum weight. | Calcium in body. | |
|---|---|---|---|---|---|---|
| | *days* | *gm.* | *gm.* | *gm.* | *gm.* | *per cent* |
| 5776 | 246 | 207 | 195 | 212 | 1.829 | 0.94 |
| 632 | 245 | 192 | 188 | 204 | 2.328 | 1.14* |
| 824 | 244 | 228 | 224 | 228 | 2.426 | 1.08 |
| 530 | 242 | 208 | 201 | 208 | 2.244 | 1.12 |
| 529 | 242 | 225 | 220 | 225 | 2.296 | 1.04 |
| 305 | 245 | 249 | 244 | 249 | 2.616 | 1.07 |
| 6120 | 245 | 224 | 219 | 224 | 2.133 | 0.97 |
| Average.. | 244 | 219 | 213 | 221 | 2.267 | 1.05 |
| 195 | 269 | 219 | 214 | 230 | 2.504 | 1.10* |
| 5774 | 258 | 203 | 198 | 211 | 2.016 | 0.96* |
| 5778 | 256 | 186 | 177 | 186 | 1.703 | 0.96 |
| Average.. | 261 | 203 | 196 | 209 | 2.074 | 1.01 |
| 159 | 321 | 240 | 236 | 240 | 2.339 | 0.99 |
| 173 | 333 | 180 | 175 | 204 | 2.276 | 1.12* |
| 197 | 346 | 184 | 180 | 188 | 1.953 | 1.08 |
| 714 | 401 | 268 | 262 | 280 | 2.651 | 0.95* |
| 638 | 426 | 255 | 245 | 255 | 2.842 | 1.16 |
| 71 | 522 | 225 | 216 | 230 | 2.549 | 1.18 |
| 251 | 537 | 245 | 244 | 255 | 2.687 | 1.05* |
| 252 | 537 | 210 | 203 | 217 | 2.615 | 1.20* |
| 3102 | 548 | 248 | 242 | 259 | 3.299 | 1.27* |
| 2893 | 596 | 270 | 264 | 270 | 2.850 | 1.08 |
| 54 | 775 | 172 | 168 | 209 | 2.705 | 1.29* |
| 182 | 880 | 144 | | 270 | 2.991 | 1.11* |
| 183 | 889 | 167 | | 280 | 2.776 | 0.99* |

* Percentage calculated on basis of maximum weight.

In contrast with this general average it was found that one female (No. 5776), which had (at the age of 8 months) raised fifteen young and whose last litter of six had been separated from her at the age of 4 weeks on the day she was killed for analysis,

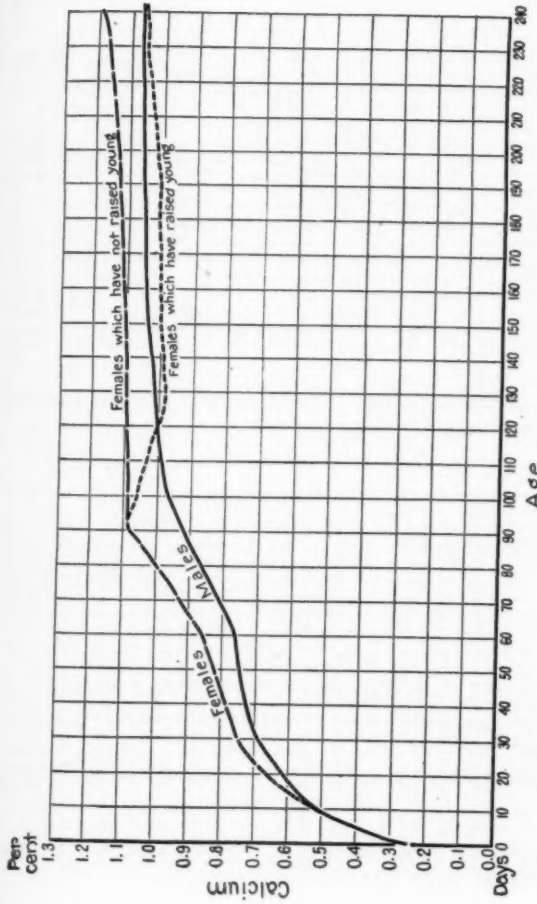


FIG. 1. Percentage of calcium on age. Showing increase in percentage of calcium in the body from birth to maturity.

showed only 1.829 gm. or 0.94 per cent of calcium. Another (No. 6120), which had raised an even larger number of young (twenty-two at 8 months) but whose last litter, of seven, had reached 4

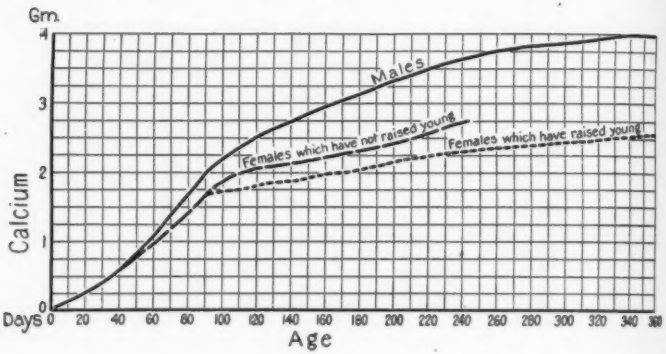


FIG. 2. Gm. of calcium on age. Showing increase in actual amount of calcium in the normal rat from birth to maturity.

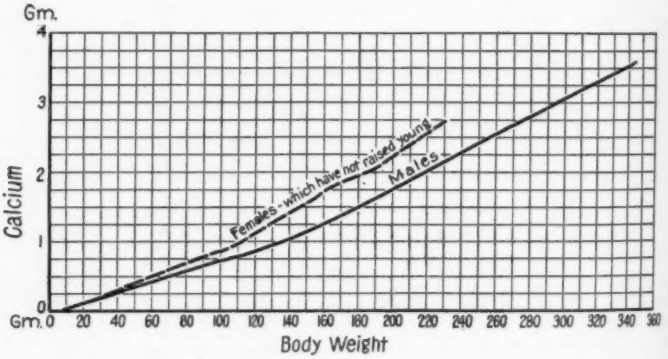


FIG. 3. Gm. of calcium on body weight. Showing relation of weight of body calcium to the body weight from birth to maturity.

weeks of age and been separated from her 12 days before she was killed for analysis, showed 2.133 gm. or 0.97 per cent of calcium. A female which had weaned her last young 6 weeks before being killed for analysis showed 1.12 per cent; and one whose young had

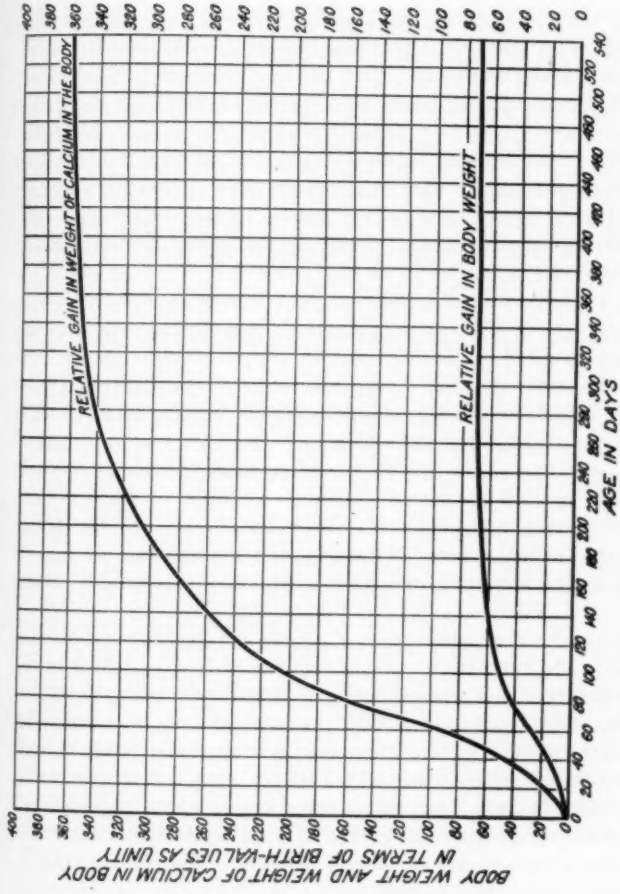
RELATIVE GAIN IN WEIGHT OF CALCIUM IN THE BODY
RELATIVE GAIN IN BODY WEIGHT
AGE IN DAYS
BODY WEIGHT AND WEIGHT OF CALCIUM IN BODY IN TERMS OF BIRTH-VALUES AS UNITY

FIG. 4. Body weight and weight of calcium in the body on age; showing gains in terms of birth values as unity.

been weaned 3 months before she was analyzed showed 1.14 per cent or nearly as much as the average of the females of the same age which had raised no young.

Thus it will be seen that the apparent losses of body calcium due to the bearing and suckling of young ranged in these individual cases from about 25 to less than 5 per cent; and when the data of individual cases are compared with each other and with the average, there appears to be a decided tendency for animals to begin to recover their normal calcium content when lactation ceases. This is in accordance with the observations of Forbes and his associates (3) upon milch cows which he found to lose body calcium during lactation and to store calcium during the intervening "dry" periods.

It is not to be assumed, however, that such recovery will always be complete. It was evidently incomplete in the following case of twin sisters killed for analysis at 269 days, whose life history had been alike except that one had reared young and the other had not: No. 196 which had not reared any young showed 2.801 gm. or 1.29 per cent of calcium; No. 195 which had reared ten young, completing the suckling of the last of them 4 months previously, showed 2.504 gm. or 1.10 per cent of calcium. Doubtless a considerable amount of calcium had been recovered, but she was still distinctly below the calcium content of her twin sister which had not reared young, and somewhat below the average for such females at her age.

From such direct comparison of strictly comparable individuals and from the averages of all our analyses of females which have, and which have not, reared young, it appears that in general the females which bore and suckled young did not so completely recover the calcium lost in the process as to reach the percentage of calcium shown by females which had not reared young; but did approximately hold their own after a loss of about one-tenth of their body calcium in the course of bearing and suckling their first one or two litters of young. Thus it will be seen in Fig. 1, that after falling from an average of about 1.1 per cent to about 1.0 per cent of body calcium, the average curve of calcium content for females which have raised young continues about parallel with (though about 10 per cent below) that for those which had not.

*Calcium in Animals on Diets Lacking Fat-Soluble Vitamin.*

Animals were analyzed which had lived from 4 weeks of age until death on a diet adequate (for rats) in all respects excepting that it contained no source of fat-soluble vitamin. The diet consisted of: Patent flour, 82 per cent; extracted meat residue, 10 per cent; Osborne and Mendel (4) salt mixture, 4 per cent; yeast, 4 per cent.

The results are shown in Table X. The percentage of calcium is calculated as stated earlier on the maximum body weight reached by the animal.

As will be seen by comparing the data of Table X with the normal curves shown in Fig. 1, the rats, which had first grown and

TABLE X.

*Calcium in Animals on a Diet Lacking Fat-Soluble Vitamin.*

| Rat No. | Sex. | Age at death. | Weight at death. | Maximum weight. | Calcium in body. | |
|---|---|---|---|---|---|---|
| | | *days* | *gm.* | *gm.* | *gm.* | *per cent** |
| 5115 | M. | 92 | 63 | 84 | 0.719 | 0.86 |
| 5187 | " | 83 | 68 | 86 | 0.686 | 0.80 |
| 5171 | " | 88 | 63 | 86 | 0.687 | 0.80 |
| 5130 | F. | 95 | 47 | 64 | 0.608 | 0.95 |
| 5172 | " | 95 | 64 | 86 | 0.743 | 0.86 |
| 5286 | " | 79 | 47 | 73 | 0.569 | 0.78 |
| Average..... | | 89 | 59 | 80 | 0.699 | 0.84 |

* Percentage calculated on the basis of maximum body weight.

then lost weight and died on a diet lacking fat-soluble vitamin, contained slightly more calcium than would be expected in normal rats of their maximum weights, and less than half as much as in normal rats of the age at which they died. The fact that these experimental animals contained somewhat more calcium than a normal animal of the same weight would suggest that some calcification of bone had continued after growth had ceased for lack of fat-soluble vitamin, or that the lack of fat-soluble vitamin in the food did not interfere with storage of calcium to as great an extent as it interfered with growth. These animals approximately doubled their body weights while on the fat-soluble vitamin-free diet. On the average they continued to grow for 5 to 6 weeks

after being placed on this diet; but their growth during this time was not at a fully average normal rate. If it had been, they would have reached maximum weights about 50 per cent higher than they actually attained. Although these rats were able to store calcium up to the normal amount for the maximum size that they reached, they never contained as much calcium as would fully normal rats of the same age. It is apparent that the animals in spite of lacking fat-soluble vitamin in their diet were able to store calcium though not at a fully normal average rate; and it is probable that the growth, and possible that the storage of calcium, may have been by virtue of the fat-soluble vitamins stored in their bodies before weaning time.

TABLE XI.

*Calcium in Animals on Diets Lacking Vitamin B.*

| Rat No. | Sex. | Age. | Weight at death. | Maximum weight. | Calcium in body. | |
|---|---|---|---|---|---|---|
| | | *days* | *gm.* | *gm.* | *gm.* | *per cent** |
| 5310 | M. | 54 | 25 | 37 | 0.437 | 1.18 |
| 5267 | " | 51 | 42 | 59 | 0.651 | 1.10 |
| 5215 | " | 64 | 41 | 59 | 0.736 | 1.25 |
| 5328 | F. | 55 | 29 | 45 | 0.524 | 1.17 |
| 5428 | " | 56 | 41 | 60 | 0.697 | 1.17 |
| 5446 | " | 56 | 35 | 52 | 0.617 | 1.19 |
| 5218 | M. | 55 | 27 | 39 | 0.459 | 1.18 |
| Average..... | | 58 | 34 | 50 | 0.589 | 1.18 |

* Percentage calculated on the basis of maximum body weight.

If the calcium found in the bodies of these animals at death were divided by their body weights at death, percentages higher than the normal for either the age or the weight would be obtained, but this would be misleading because of the fact that death was preceded by large losses in body weight which cannot be conceived of as accompanied by corresponding losses of calcium.

*Calcium in Animals on Diets Lacking Vitamin B.*

Table XI shows data for seven rats which received from the age of 28 days until death a diet adequate in all other respects, but lacking vitamin B. It will be seen that they survived an average of 28 days on this diet, the average age at death being 56 days.

The average weight at death was 34 gm. or rather less than when placed on this diet at 28 days. Here, as in the case of diet lacking vitamin A, there is typically a rapid loss of body weight before death and there is no reason to suppose that this is accompanied by a corresponding loss of body calcium, so that to divide the weight of calcium found in the body by the body weight at death would give a misleadingly high percentage. Dividing the average weight of body calcium by the average maximum body weight yields 1.18 per cent of calcium which is a slightly higher percentage than is found in normal rats of the same age, but this also is likely to be misleading unless it be kept in mind that the reason for this high percentage is that even the maximum body weight of these animals was much below the normal for their age. The animals, although supplied abundantly with fat-soluble vitamins, since they received *ad libitum* a diet containing 2 per cent of cod liver oil, did not store calcium in so large amounts as do normally fed rats of their age.

It will be observed that both the diet lacking fat-soluble vitamins and that lacking vitamin B contained adequate proportions of calcium and other mineral elements and were excellently constituted for the nutrition of rats in all respects except the specific vitamin deficiencies noted. In each case as the vitamin deficiency checked the normal growth of the body it also prevented the body from storing as large an amount of calcium as it would have stored during the same time in making a normal growth upon a normal diet. In both cases the amount of calcium which the animals actually contained lay between the amount which would be normal for their weight and the amount which would be normal for their age.

*Calcium in Animals on Diets Lacking Sufficient Protein (Cystine the First Limiting Factor).*

As described elsewhere (5) five male rats of the same litter were fed on a mixture consisting essentially of five-sixths starch and one-sixth whole milk powder with a small amount of yeast as basal ration, to which was added in one case ferric citrate, in another butter fat, in a third cystine, two of the rats receiving the basal ration only. Only the rat receiving the cystine made normal growth, and he alone contained a normal weight of body calcium

for his age. The other four, whose growth had been decidedly stunted by shortage of cystine, all contained decidedly smaller amounts of calcium, but slightly higher percentages. Here it is evident that, with growth greatly retarded by overdilution of a qualitatively adequate food protein by starch (the specific deficiency being shortage of cystine), the calcification of the bones and consequent increase of body calcium is not retarded in the same degree as is the increase in body weight. The data are shown as "First series" in Table XII).

TABLE XII.

*Calcium in Animals on Diets with Cystine as Limiting Factor.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| First series. | | | | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 333 | 255 | 181 | 178 | 2.019 | 1.13 |
| 334 | 255 | 161 | 165 | 1.005 | 1.16 |
| 335 | 255 | 275 | 271 | 2.721 | 1.00 |
| 336 | 255 | 136 | 134 | 1.468 | 1.10 |
| 337 | 255 | 172 | 171 | 1.954 | 1.14 |
| Second series. | | | | | |
| 379 | 255 | 112 | 110 | 1.189 | 1.17 |
| 380 | 255 | 135 | 134 | 1.646 | 1.22 |
| 381 | 255 | 125 | 120 | 1.462 | 1.19 |
| 382 | 255 | 112 | 110 | 1.515 | 1.38 |
| 383 | 255 | 207 | 204 | 2.148 | 1.05 |
| 384 | 255 | 210 | 208 | 2.459 | 1.18 |
| 387 | 255 | 142 | 141 | 1.564 | 1.11 |
| 388 | 255 | 132 | 131 | 1.644 | 1.26 |

In another, similar, experiment ("Second series" in Table XII), four animals (Nos. 379, 381, 383, and 387) received respectively the basal diet just described and the same plus additions of butter fat, cystine, or ferric citrate. Four other animals (Nos. 380, 382, 384, and 388) received the same diets respectively except for the addition of 1 per cent of calcium carbonate. This addition of calcium carbonate to the food did not increase the rate of growth in body weight, but did increase somewhat the storage of body calcium so that the average percentage of calcium in the bodies of these four rats became 1.27 as compared with 1.13 per cent in the

bodies of the four animals receiving diets otherwise parallel but without the added calcium carbonate.

In the second series as in the first, the rats receiving added cystine and making better growth gained larger amounts of body calcium but not in proportion to the gains in body weight so that the percentages of calcium were less. The rats receiving the cystine and growing at a rate somewhat approaching the normal showed about normal percentages of body calcium; while those greatly stunted in growth by shortage of cystine were not retarded in storage of calcium to the same extent as in growth and thus acquired a percentage of body calcium somewhat above the normal although the actual amounts of calcium contained in their bodies were much below the amounts found in normal rats of the same age.

In all of the cases in which growth was seriously interfered with by quantitative deficiency of protein (shortage of cystine), as in the cases of lack of vitamin A or of vitamin B, the absolute amounts of body calcium found have been less than the amounts contained in normal rats of the same age and more than the amounts contained in normal rats of the same body weight.

This we have also found to be true of animals which were placed when 4 weeks of age upon diets consisting of whole wheat and whole milk mixtures diluted with starch to an extent calculated to permit of bare maintenance of body weight. Such animals slowly increased their calcium content so that at 90 days of age they contained about twice as much calcium as at 28 days.

*Calcium Content of Animals Which Have Been Kept on Low Calcium Diets.*

Voit (6) by experiments upon three young dogs, one killed at the beginning and analyzed as control, one fed on a diet poor in calcium, and a third on the same diet with the addition of bone ash, showed that the animal on the low calcium diet actually lost body calcium while the one receiving the bone ash gained. Similarly we have found that rats placed at 28 days of age upon a diet of flour and sodium chloride and surviving without appreciable growth to an average age of about 80 days showed no increase in body calcium as compared with the normal for 28 days, but rather a slight decrease.

Rats which have first been allowed to make normal growth to a larger size and higher calcium content and are then placed on a

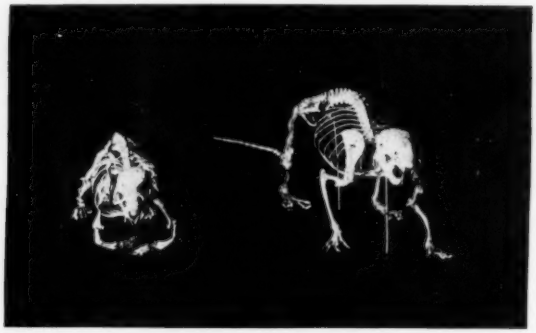

FIG. 5.

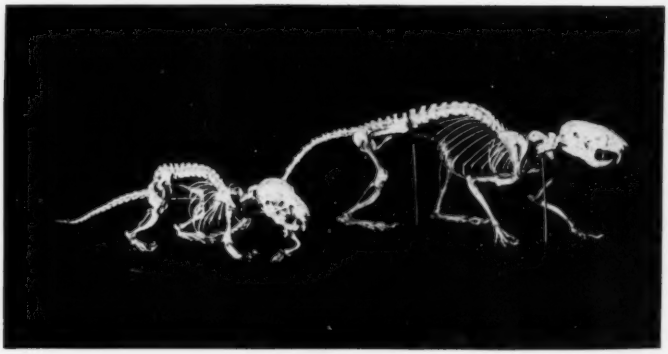

FIG. 6.

FIGS. 5 and 6. Front and side views of skeletons of twin brothers (albino rats), one of which had received a diet of normal calcium content (wheat, meat, and milk) while the other had received a low calcium diet (wheat and meat).

similarly deficient diet can readily be shown to lose relatively large amounts of their body calcium. Several such animals studied by us have shown at death only two-thirds to three-

fourths of the amounts of calcium which they must have contained when transferred from normal food to calcium-deficient diet; and in one of these cases (No. 1767) which has been mentioned by Sherman and Kramer in connection with studies of vitamin A (7), a rat which survived on a diet very low in calcium for about 7 months was found to have lost nearly one-half of the calcium which it presumably contained at the beginning of this experiment.

Toverud (8), in a paper published while our work was in progress, has likewise shown that calcium-poor diet may result in an actual loss of body calcium, from the teeth as well as from the bones.

Osborne and Mendel have shown (9) that when young growing animals (rats) are placed upon a diet adequate in other respects but very low in calcium, some are promptly checked in growth while others grow for some time before showing any outwardly visible effect of the calcium deficiency.

We have encountered similar individual variations among animals subsisting upon natural food materials of low calcium content. One rat placed when 28 days of age upon a diet of wheat and meat was able to grow from 40 to 128 gm. Calculated on the latter weight, this animal contained only 0.52 per cent of calcium or much less calcium than either a normal rat of his weight or a normal rat of his age. Another rat of the same litter and sex which had received the same wheat and meat but also milk in the diet contained 1.05 per cent of calcium at the same age. The skeletons of duplicates of these two rats are shown in Figs. 5 and 6.

*Effects of the Addition of Cod Liver Oil or of Calcium to a Diet of Natural Foods Which Had Been Found Adequate for Growth and Reproduction but Not for Optimal Storage of Calcium in the Body.*

As explained above, the animals analyzed for the establishment of the normal calcium content of the body at different ages were from families whose dietaries contained whole milk powder to the extent of about one-third of the weight of the total food solids (Diet B).

Other families of rats in this laboratory have subsisted for several successive generations upon an exclusive diet of one-sixth whole milk powder and five-sixths ground whole wheat (Diet A). This diet is only slightly over one-half as rich in calcium or in fat-

soluble vitamin as is that from which the rats were taken for the determination of normal calcium content at different ages, and it does not support either growth or reproduction with as high an efficiency (10); yet as it has supported normal nutrition including the extra demands of reproduction and lactation successfully through many successive generations it must be regarded as an adequate although not an optimal diet. It was therefore of interest to determine the calcium content of rats from families maintained on Diet A. It was found that they contained, at each of the ages studied, somewhat less calcium both in absolute amounts and in percentages of their body weights, than the parallel animals from families of the same stock which had been fed on diets richer in calcium and fat-soluble vitamin.

Since much recent work has indicated that a fat-soluble vitamin or antirachitic factor contained in cod liver oil may promote calcification in the bones of young animals on calcium-poor food, it was decided to try the effect, upon the storage of calcium in the body, of adding cod liver oil or calcium lactate to the diet of lower calcium and fat-soluble vitamin content (Diet A).

Litters of young rats were divided at the age of 28 days, some placed upon Diet A, some upon Diet A plus cod liver oil, and some upon Diet A plus calcium lactate. All other conditions were maintained strictly uniform. Table XIII shows the results obtained on killing such rats and determining their calcium content at the age of 60 days; Table XIV shows the results of similar experiments continued to an age of 90 days.

The results show that cod liver oil added to Diet A made no appreciable difference in the percentage of calcium in the rat at either 60 or 90 days. (Since at 90 days the weight of the males on Diet A plus cod liver oil was slightly higher than the weight of the males on Diet A, the average weight of calcium is greater. This difference in weight is marked in the males. In every case the animal on Diet A plus cod liver oil weighed more than its litter control. This difference was not found for the females.)

The addition of calcium lactate to the same diet increased the percentage of calcium in the body up to the normal figure. The growth of the males to 90 days on Diet A plus calcium lactate was also somewhat better than on Diet A alone; but the animals on both Diet A plus cod liver oil and on Diet A plus calcium lactate

TABLE XIII.

*Calcium in 60 Day Old Rats on Diet A, Diet A Plus 1 Per Cent Cod Liver Oil, and Diet A Plus 1 Per Cent Calcium Lactate.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| | | Males. | | | |
| | | Diet A. | | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 10018 | 60 | 106 | 102 | 0.645 | 0.63 |
| 9645 | 62 | 119 | 114 | 0.739 | 0.65 |
| 9637 | 62 | 115 | 110 | 0.621 | 0.57 |
| 12862 | 60 | 123 | 116 | 0.693 | 0.60 |
| 9158 | 60 | 133 | 127 | 0.808 | 0.64 |
| 9224 | 61 | 128 | 126 | 0.842 | 0.67 |
| 9226 | 61 | 98 | 95 | 0.600 | 0.63 |
| Average... | 61 | 117 | 113 | 0.707 | 0.63 |
| | | Diet A plus 1 per cent cod liver oil. | | | |
| 10019 | 60 | 118 | 112 | 0.683 | 0.61 |
| 9644 | 62 | 141 | 135 | 0.870 | 0.64 |
| 9638 | 62 | 91 | 84 | 0.556 | 0.66 |
| 12861 | 60 | 129 | 121 | 0.737 | 0.61 |
| 9159 | 60 | 158 | 150 | 0.912 | 0.61 |
| 9225 | 61 | 124 | 121 | 0.740 | 0.61 |
| 9227 | 61 | 124 | 121 | 0.730 | 0.60 |
| Average... | 61 | 126 | 121 | 0.747 | 0.62 |
| | | Diet A plus 1 per cent calcium lactate. | | | |
| 10020 | 60 | 111 | 105 | 0.860 | 0.82 |
| | | Females. | | | |
| | | Diet A. | | | |
| 10023 | 60 | 94 | 91 | 0.556 | 0.61 |
| 9020 | 61 | 102 | 98 | 0.710 | 0.72 |
| 8754 | 67 | 133 | 125 | 0.855 | 0.68 |
| 12863 | 60 | 118 | 112 | 0.765 | 0.68 |
| 12866 | 60 | 111 | 104 | 0.707 | 0.68 |
| 9161 | 60 | 102 | 99 | 0.710 | 0.72 |
| Average... | 61 | 110 | 105 | 0.716 | 0.68 |

TABLE XIII—*Concluded.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| Diet A plus 1 per cent cod liver oil. | | | | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 10022 | 60 | 105 | 100 | 0.635 | 0.64 |
| 9022 | 61 | 112 | 107 | 0.638 | 0.60 |
| 8756 | 67 | 144 | 140 | 0.916 | 0.65 |
| 12864 | 60 | 114 | 108 | 0.712 | 0.71 |
| 12865 | 60 | 106 | 101 | 0.705 | 0.70 |
| 9160 | 60 | 123 | 116 | 0.744 | 0.67 |
| Average... | 61 | 117 | 112 | 0.730 | 0.66 |
| Diet A plus 1 per cent calcium lactate. | | | | | |
| 10021 | 60 | 114 | 111 | 0.925 | 0.83 |

were below the average normal weight. Therefore, although the animals which received an addition of calcium in their diet had the same percentage of calcium in their bodies as the average normals, they contained less total weight of calcium.

Increasing the fat-soluble vitamin in the diet, therefore, did not aid in the deposition of calcium, but when calcium lactate was added to the same diet there was an increase in the calcium content of the animal. Bethke, Steenbock, and Nelson (11) found, with the phosphorus content of the diet constant, that on increasing the calcium and the fat-soluble vitamin of the diet simultaneously, the calcium content of the bone increased up to a certain point only, beyond which addition of either or both had no further effect. Interpreting the results reported here in the light of the findings of these authors, it would appear that there was enough of the "antirachitic factor" in the whole milk powder of Diet A to promote calcification if sufficient calcium were present, since increasing this factor in the diet did not increase calcification, whereas without additional vitamin but with increased calcium the percentage of calcium was brought up to normal.

Diet A differed from the food of the rats analyzed as "normals" (Tables I to IX) not only in the calcium and fat-soluble vitamin content as noted above, but also in containing a slightly lower percentage of protein which in turn was probably also a some-

TABLE XIV.

*Calcium in 90 Day Old Rats on Diet A, Diet A Plus 1 Per Cent Cod Liver Oil, and Diet A Plus 1 Per Cent Calcium Lactate.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| | | Males. | | | |
| | | Diet A. | | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 10143 | 91 | 163 | 158 | 1.112 | 0.70 |
| 10222 | 90 | 178 | 171 | 1.296 | 0.76 |
| 10274 | 90 | 178 | 172 | 1.215 | 0.71 |
| 8713 | 90 | 225 | 218 | 1.581 | 0.73 |
| 9057 | 90 | 150 | 146 | 1.071 | 0.73 |
| 9164 | 90 | 150 | 144 | 0.936 | 0.65 |
| 9171 | 90 | 198 | 191 | 1.376 | 0.72 |
| 9257 | 90 | 174 | 168 | 1.162 | 0.69 |
| 2426 | 90 | 132 | 127 | 0.801 | 0.63 |
| 2427 | 90 | 124 | 118 | 0.781 | 0.66 |
| 2428 | 90 | 122 | 117 | 0.866 | 0.74 |
| Average.. | 90 | 163 | 157 | 1.109 | 0.70 |
| | | Diet A plus 1 per cent cod liver oil. | | | |
| 10142 | 91 | 240 | 235 | 1.617 | 0.69 |
| 10148 | 92 | 208 | 203 | 1.422 | 0.70 |
| 10220 | 90 | 215 | 209 | 1.524 | 0.73 |
| 10276 | 90 | 200 | 194 | 1.375 | 0.71 |
| 10277 | 90 | 185 | 179 | 1.231 | 0.69 |
| 8711 | 90 | 241 | 232 | 1.676 | 0.72 |
| 9059 | 90 | 199 | 196 | 1.335 | 0.68 |
| 9058 | 90 | 209 | 205 | 1.421 | 0.69 |
| 9018 | 92 | 210 | 205 | 1.318 | 0.64 |
| 9165 | 90 | 201 | 195 | 1.295 | 0.66 |
| 9258 | 90 | 191 | 186 | 1.342 | 0.72 |
| 9170 | 90 | 235 | 228 | 1.565 | 0.69 |
| 9169 | 90 | 225 | 218 | 1.491 | 0.67 |
| 9256 | 90 | 206 | 200 | 1.455 | 0.73 |
| Average... | 90 | 212 | 206 | 1.433 | 0.69 |
| | | Diet A plus 1 per cent calcium lactate. | | | |
| 10141 | 91 | 210 | 203 | 1.862 | 0.92 |
| 10149 | 92 | 204 | 199 | 1.796 | 0.90 |
| 10221 | 90 | 191 | 183 | 1.753 | 0.96 |
| 10275 | 90 | 180 | 175 | 1.601 | 0.92 |
| 10279 | 90 | 174 | 170 | 1.530 | 0.90 |
| Average... | 91 | 192 | 186 | 1.708 | 0.92 |



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TABLE XIV—*Concluded.*

| Rat No. | Age. | Last live weight. | Net weight. | Calcium in body. | |
|---|---|---|---|---|---|
| | | Females. | | | |
| | | Diet A. | | | |
| | *days* | *gm.* | *gm.* | *gm.* | *per cent* |
| 9233 | 90 | 125 | 121 | 0.968 | 0.80 |
| 10150 | 92 | 153 | 148 | 1.208 | 0.82 |
| 10223 | 90 | 153 | 147 | 1.159 | 0.79 |
| 10145 | 91 | 137 | 133 | 1.049 | 0.79 |
| 10284 | 90 | 130 | 126 | 1.130 | 0.90 |
| 8714 | 90 | 171 | 166 | 1.392 | 0.84 |
| 8760 | 90 | 138 | 133 | 1.036 | 0.78 |
| 7384 | 90 | 101 | 98 | 0.745 | 0.76 |
| 7386 | 90 | 108 | 104 | 0.767 | 0.74 |
| Average... | 90 | 135 | 130 | 1.050 | 0.80 |
| | | Diet A plus 1 per cent cod liver oil. | | | |
| 10152 | 92 | 147 | 142 | 1.163 | 0.82 |
| 10225 | 90 | 154 | 154 | 1.223 | 0.83 |
| 10144 | 91 | 157 | 152 | 1.127 | 0.74 |
| 10286 | 90 | 149 | 145 | 1.288 | 0.89 |
| 10288 | 90 | 104 | 99 | 0.744 | 0.75 |
| 8758 | 90 | 134 | 124 | 0.947 | 0.76 |
| 9231 | 90 | 150 | 146 | 1.259 | 0.86 |
| Average... | 90 | 142 | 137 | 1.107 | 0.81 |
| | | Diet A plus 1 per cent calcium lactate. | | | |
| 10151 | 92 | 148 | 144 | 1.544 | 1.07 |
| 10224 | 90 | 153 | 147 | 1.562 | 1.06 |
| 10285 | 90 | 135 | 131 | 1.429 | 1.09 |
| 10287 | 90 | 138 | 134 | 1.437 | 1.07 |
| Average... | 91 | 144 | 139 | 1.493 | 1.07 |

what less efficient protein mixture since it was derived more largely from seed and less largely from milk or meat. Other differences between the diets which might be pointed out are doubtless of very minor if any significance.

In view of the fact that increasing the calcium content of Diet A was sufficient, without other change, to induce normal storage

of calcium in the young growing animals, it became unnecessary to investigate the possible effects of other enrichments of the diet in this connection.

In view of the large amount of evidence of the presence of a "calcification factor" in cod liver oil, and our own observations recorded elsewhere of the marked influence of a liberal intake of fat-soluble vitamin upon family vigor (12), it is somewhat striking to find here such a complete success in the promotion of calcification by the addition of calcium to the diet and no appreciable effect from the addition of cod liver oil. We believe that it is well to emphasize strongly the importance of the fat-soluble vitamins in nutrition, but evidently for optimal storage of calcium in the normal young even a liberal allowance of cod liver oil was less effective than the simple increase in the calcium content of the food. In the experiments here described the increase was from 0.20 to 0.33 per cent of calcium in the air-dry food mixture.

## SUMMARY.

The calcium content of the normal white rat has been established by analyses of large numbers of animals at definite ages.

In round numbers the percentage of calcium in the body is found to increase from 0.25 per cent at birth to about 0.6 per cent at 15 days; about 0.7 per cent at 30 days; 0.75 to 0.85 per cent at 60 days; 0.95 to 1.1 per cent at 90 days; and 1.0 to 1.2 per cent in the adult animal.

Females which had not raised young showed higher percentages of calcium than males of the same ages and inheritance which had received the same food.

Beginning with 60 days, the gross weight of calcium in the body was higher for males than for females since the average weight of the males was greater.

The increase in weight and percentage of calcium was found to be rapid up to 90 days. Both for males and for females which have not raised young, there is a slower but steady increase from 90 days to 8 months. Between the ages of 8 and 12 months in the males the slow increase continued.

For the females the results are more difficult to summarize beyond the age of 90 days because of the different breeding records. Before the age of 120 days there usually occurs a distinct lowering

of the percentage of calcium in the body as the result of the bearing and suckling of young. In the average there was found a lowering of the percentage of body calcium by about one-tenth after which there appeared to be an approximate balance between the tendency to lose body calcium during the lactation periods and the tendency to regain it during the intervening periods.

Using, as a basis of comparison and interpretation, the standard data obtained by the above analyses of normal rats of both sexes and different ages, the effects of several different dietary deficiencies upon the storage of calcium in the young have been investigated.

Animals which had received ample calcium but were stunted in growth because of other deficiencies in the diet—lack of vitamin A or B, or too little protein (shortage of cystine)—in all cases had higher calcium content than normal rats of the same weight, but less calcium than normal animals of the same age.

Some animals were stunted by feeding upon a diet consisting of an originally satisfactory mixture of wheat and milk which had been overdiluted with starch to such an extent that the rats eating *ad libitum* of the diet were barely maintained at constant weight when they should have been growing rapidly on the same diet without the starch dilution. This form of dietary stunting also resulted in a calcium content of the body between the amount which would have been normal for the age and the amount which would have been found in a (much younger) normal rat of the same body weight.

Animals which receive sufficiently little calcium in their diets lose calcium from their bodies. Animals placed on such a diet at weaning show this result as well as animals which have been transferred when older from a normal diet. If an animal is able to grow on such a low calcium diet its calcium content falls below the normal for its weight as well as for its age.

In another series of experiments it was found that animals on a diet of one-sixth whole milk powder and five-sixths wheat (Diet A) have a lower percentage of calcium in their bodies than animals on a diet of one-third whole milk powder and two-thirds wheat (Diet B). Here, as in most cases of suboptimal nutrition encountered in human experience, there is more than one dietary factor which might conceivably have had an influence in bringing about

the observed result. The two factors which seemed most likely to be influential were tested separately by means of feeding experiments with cod liver oil and with added calcium as lactate. Addition of 1 per cent of cod liver oil to the former diet (Diet A) did not raise the percentage of calcium in the body; whereas when 1 per cent of calcium lactate was added to this diet the percentage of calcium in the body was raised to the normal figure. Without questioning the importance of the vitamin factor in certain relations, it is plain that in cases such as those here studied the factor of prime importance to the optimal storage of calcium in the growing body is the presence of a sufficiently liberal allowance of calcium in the food.

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# THE BLOOD SUPPLY TO THE PANCREAS WITH SOME PERFUSION STUDIES.

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I.

## *Blood Supply to the Pancreas.*

An anatomical study of the circulation of the pancreas was undertaken with the view of determining the arteries going directly to the organs so that an exact knowledge could be had of the direction taken by any perfused fluids.

The method employed in these studies was a modification of Gross' technique (1) for injecting the coronary vessels of the heart. In order to simulate the normal condition in the living animal it was necessary to use proper temperature and pressure controls in injecting the vessels. The apparatus devised for this work is shown diagrammatically (Fig. 1). The animal was first exsanguinated by bleeding the jugular vein, then the thoracic aorta was exposed and a glass cannula inserted proximal to its entrance through the diaphragm. The Woulfe bottles $B$ and $C$ were submerged in the copper tank and the water maintained at a temperature of 42–45°C. Clamps $H_1$ were opened and the saline solution was permitted to flow at a pressure of 120 to 140 mm. When the washings (drained from the inferior vena cava) came clear the $H_1$ clamps were closed and the $H_2$ clamps opened, thus permitting the barium gelatin mass to flow through. When the arteries were completely filled—determined by the pressure rising above 150 mm.—the cannula was removed, the aorta ligated, and the animal placed in the ice box to cool. It will be observed that no abdominal incision was made and that there was no form of manipulation to disturb the normal relation of the abdominal



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viscera. The entire injection was conducted through the thoracic aorta. After the gelatin had hardened the abdominal viscera were removed *in toto*; then the pancreas and its blood supply were carefully dissected. A typical X-ray photograph of one of these specimens is presented in Fig. 2. The specimen was then dehydrated in alcohol, starting with 50 per cent and going through absolute, and finally cleared in methyl salicylate by the method of Spalteholz (2) (Fig. 3). The entire pancreas and its arterial

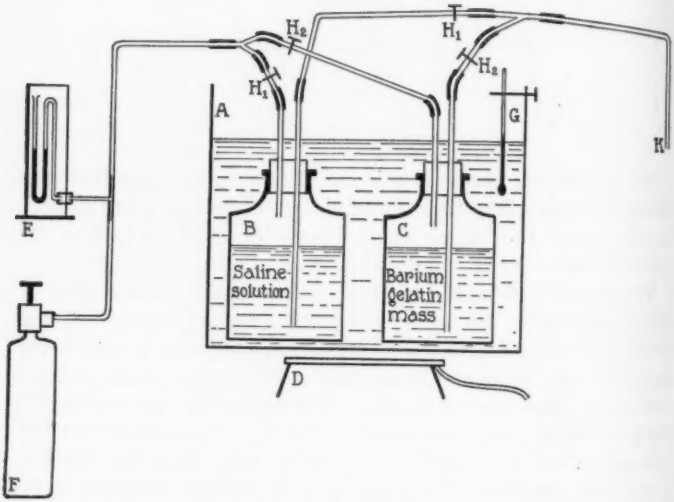


FIG. 1. *A*, copper tank; *B*, Woulfe bottle with saline solution; *C*, Woulfe bottle with barium gelatin mass; *D*, gas burner; *E*, manometer; *F*, oxygen tank; *G*, thermometer; $H_1$, clamps controlling saline solution flow; $H_2$, clamps controlling barium flow; *K*, cannula in thoracic aorta.

supply can be visualized in the X-ray photograph. Fig. 2 shows the hepatic artery (3), arising from the celiac axis, first giving off the right gastric, then the gastroduodenal artery. Its branches continue upward as the two terminal hepatic arteries, the sole arterial supply to the liver. The gastroduodenal artery turns down toward the pylorus and, after giving off the small right gastroepiploic artery, continues on as the superior pancreaticoduodenal artery to supply the neck and a portion of the body of

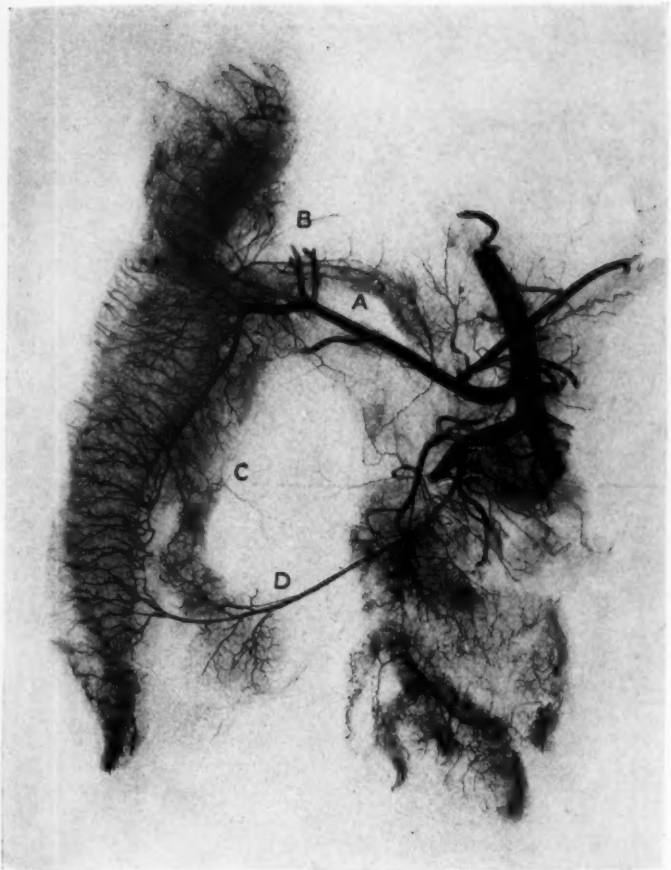


FIG. 2. X-ray photograph of pancreas and duodenum, showing arteries injected with barium gelatin mass. Hepatic artery (*A*) arising from the celiac axis and giving off two terminal hepatic arteries (*B*) upward toward the liver, then proceeding as the superior pancreaticoduodenal artery (*C*). Also the inferior pancreaticoduodenal artery (*D*).

FIG. 3. Photograph of same specimen as Fig. 2 cleared in methyl salicylate.

FIG. 4. X-ray photograph showing rich anastomosis between superior and inferior pancreaticoduodenal artery—after injecting the superior pancreaticoduodenal artery.

the pancreas, together with the first portion of the duodenum. The inferior pancreaticoduodenal artery arises from the superior mesenteric and supplies the head of the pancreas and the second portion of the duodenum. A rich anastomosis can be seen in both the duodenum and pancreas between the superior and inferior pancreaticoduodenal arteries (Fig. 4). (Only the superior pancreaticoduodenal artery was injected.) The body and tail of the pancreas are seen on dissection to be supplied by several small branches arising from the splenic artery. The veins drain-

TABLE I.

*Effect of Injecting Saline Solution into Portal Vein and Hepatic Artery.*

Dog 51. Weight, 6.5 kilos. Amytal, 3.9 cc. Feb. 16, 1925.

| Time. | Procedure. | Sugar. | |
|---|---|---|---|
| | | In blood. | In urine.* |
| *a.m.* | | *mg.* | *gm.* |
| 10.40 | Operation started. | | |
| 10.55 | | 0.107 | 0 |
| 11.03 | Injected 10 cc. saline solution into portal vein. | | |
| 11.10 | | 0.131 | |
| 11.35 | | | 0 |
| 11.55 | | 0.134 | 0 |
| *p.m.* | | | |
| 12.10 | Injected 10 cc. saline solution into hepatic artery. | | |
| 12.20 | | 0.188 | |
| 12.40 | | | + |
| 1.20 | | 0.121 | + |

* Retention catheter in bladder.

ing the pancreas all enter the portal system. One may observe that the arteries furnishing the most practical approach for direct perfusion of the pancreas are the superior and inferior pancreaticoduodenals.

II.

*Studies of Saline Perfusion through the Arteries of the Pancreas and Liver.*

Perfusion studies of these arteries were undertaken with the view of determining their effect upon the sugar excretion in the

urine and on the blood sugar level. Dogs were employed in all the experiments. Amytal (4), which has been shown to have no effect upon the blood sugar, was the anesthetic of choice, using 0.6 cc. of a 10 per cent solution per kilo of body weight. The Shaffer-Hartmann method of blood sugar determination was employed, and the Benedict test for the urine. 10 cc. of normal saline solution at 40°C. were used in all the perfusion experiments.

Typical results are seen in Tables I to VI. Injection of saline

TABLE II.

*Effect of Saline Solution in Hepatic Artery.*

Dog 55. Weight, 7.2 kilos. Mar. 4, 1925.

| Time. | Procedure. | Sugar. | |
|---|---|---|---|
| | | In blood. | In urine.* |
| *a.m.* | | *mg.* | *gm.* |
| 10.15 | Administered 4.1 cc. amytal. | | |
| 10.45 | Operation started. | | |
| 11.30 | | 0.113 | 0 |
| 11.36 | Injected 10 cc. saline solution into hepatic artery; superior pancreaticoduodenal artery ligated. | | |
| 11.39 | | | + |
| 11.41 | | 0.114 | |
| *m.* | | | |
| 12.00 | | 0.113 | |
| *p.m.* | | | |
| 12.10 | | | + |
| 12.40 | | 0.111 | + |
| 1.40 | | 0.114 | Slight. |

* Retention catheter in bladder.

sol[illegible] the hepatic artery caused an immediate glycosuria, with a slight elevation in blood sugar (Table I).

Injection of the hepatic artery with the superior pancreaticoduodenal artery completely ligated, thus causing the saline solution to flow only through the liver, produced a similar glycosuria (Table II).

By perfusing the superior pancreaticoduodenal artery with the terminal hepatic arteries tied off, so that the fluid was directed through the pancreas and not the liver, neither hyperglycemia nor

glycosuria took place. When, in the same animal, the superior pancreaticoduodenal artery was ligated and the hepatics perfused, thus changing the course of the perfusing fluid through the liver, there was an immediate rise in the blood sugar and sugar appeared in the urine (Table III).

TABLE III.

*Effect of Saline Solution in Superior Pancreaticoduodenal and Hepatic Arteries.*

Dog 56. Weight, 4.6 kilos. Mar. 11, 1925.

| Time. | Procedure. | Sugar. | |
|---|---|---|---|
| | | In blood. | In urine.* |
| a.m. | | mg. | gm. |
| 10.00 | Administered 2.8 cc. amytal. | | |
| 10.30 | | | 0 |
| 10.40 | Operation started. | | |
| 11.00 | | | 0 |
| 11.07 | | 0.089 | |
| 11.09 | | | 0 |
| 11.15 | Injected 10 cc. saline solution into superior pancreaticoduodenal artery; hepatic artery ligated. Anemia of pancreas and marked peristalsis of duodenum. | | |
| 11.20 | | 0.089 | |
| 11.25 | | | 0 |
| 11.40 | | 0.096 | 0 |
| m. | | | |
| 12.00 | | | 0 |
| p.m. | | | |
| 12.20 | | 0.098 | 0 |
| 12.25 | Injected 10 cc. saline solution into hepatic artery. | | |
| 12.28 | | 0.104 | |
| 12.30 | | | Trace |
| 12.36 | | | + |
| 1.00 | | 0.110 | + |

* Retention catheter in bladder.

Knowing that the inferior pancreaticoduodenal artery has no relation to the arterial supply of the liver, but goes only to the pancreas and duodenum, this artery was perfused without effecting

any change in the blood sugar, and no sugar appeared in the urine (Tables IV and V).

On the other hand, perfusion of saline solution through the portal vein does produce rises in blood sugar and the appearance of traces of sugar in the urine. The reaction to the perfusion seems, however, to be delayed and much less marked than similar perfusion through the arterial circulation of the liver (Tables I, V, and VI).

Epstein and Rosenthal (5), in an extensive series of experiments, found that when they perfused the arterial blood supply to the

TABLE IV.

*Effect of Saline Solution in Inferior Pancreaticoduodenal Artery.*

Dog 54. Weight, 8.8 kilos. Feb. 27, 1925.

| Time. | Procedure. | Sugar. | |
|---|---|---|---|
| | | In blood. | In urine.* |
| *a.m.* | | *mg.* | *gm.* |
| 10.10 | Administered 5 cc. amytal. | | |
| 10.55 | Operation started. | | 0 |
| 11.40 | | 0.109 | |
| 11.42 | | | 0 |
| 11.55 | Injected 10 cc. saline solution into inferior pancreaticoduodenal artery. | | |
| *p.m.* | | | |
| 12.02 | | 0.107 | |
| 12.05 | | | 0 |
| 12.30 | | 0.106 | 0 |
| 1.15 | | 0.111 | |
| 1.20 | | | 0 |
| 2.35 | | | 0 |

* Retention catheter in bladder.

pancreas, hyperglycemia and glycosuria occurred. They interpreted these experimental results as indicating that the process of perfusion in some way caused trypsin formed in the pancreas to inactivate insulin, with a consequent production of the diabetic condition. They concluded that this reaction between trypsin and insulin constituted an important etiological factor in the production of diabetes.

Repetition of these experiments of Epstein by the perfusion of saline solution into the hepatic artery of the dog confirmed his

original observations, but when the two terminal hepatic arteries were ligated the pancreas could then be perfused without producing either a hyperglycemia or glycosuria. On the contrary, when the superior pancreaticoduodenal artery was ligated with the hepatic branches patent, perfusion resulted in glycosuria, with an elevation

TABLE V.

*Effect of Saline Solution in Inferior Pancreaticoduodenal Artery and Portal Vein.*

Dog 58. Weight, 4.5 kilos.

| Time. | Procedure. | Sugar. | |
|---|---|---|---|
| | | In blood. | In urine.* |
| *a.m.* | | *mg.* | *gm* |
| 10.15 | Administered 3 cc. amytal. | | |
| 10.30 | Operation started. | | |
| 10.57 | | | 0 |
| 11.20 | | | 0 |
| 11.24 | Injected 10 cc. saline solution into inferior pancreaticoduodenal artery with portal vein clamped. | | |
| 11.25 | | 0.075 | |
| 11.35 | | 0.071 | 0 |
| 11.50 | | | 0 |
| *m.* | | | |
| 12.00 | | 0.075 | 0 |
| *p.m.* | | | |
| 12.10 | | | 0 |
| 12.27 | | 0.074 | 0 |
| 12.32 | Injected 10 cc. saline solution into portal vein. | | |
| 12.34 | | 0.090 | |
| 12.40 | | | Slight. |
| 1.00 | | | 0 |
| 1 10 | | 0.089 | |
| 1.30 | | | 0 |

* Retention catheter in bladder.

in the blood sugar. This clearly indicates that the effect is upon the liver and not upon the pancreas. Furthermore, perfusion of the inferior pancreaticoduodenal artery, which has no relation to the arterial supply of the liver, has no effect whatever upon the blood sugar and produces no glycosuria.

It is interesting to note that a marked glycosuria can be pro-

duced by arterial perfusion of the liver with saline solution, without causing a hyperglycemia. The blood sugar has even been found to stay at the same level as the control in some cases.

It seems, also, that the blood sugar balance and attending glycosuria are much more easily disturbed by perfusing the liver through the hepatic artery than through the portal vein,

TABLE VI.

*Effect of Injecting Saline Solution into Portal Vein and Hepatic Artery.*

Dog 52. Weight, 5.7 kilos. Amytal, 3.5 cc. Feb. 20, 1925.

| Time. | Procedure. | Sugar. | |
|---|---|---|---|
| | | In blood. | In urine.* |
| *a.m.* | | *mg.* | *gm.* |
| 11.15 | Operation started. | | 0 |
| 11.35 | | 0.100 | |
| 11.38 | Injected 10 cc. saline solution into portal vein. | | 0 |
| 11.45 | | | 0 |
| 11.48 | | 0.091 | |
| *p.m.* | | | |
| 12.10 | | | + |
| 12.12 | | 0.094 | |
| 12.15 | Superior pancreaticoduodenal artery tied off. | | |
| 12.42 | Injected 10 cc. saline solution into hepatic artery. | | |
| 12.43 | | | + |
| 12.44 | | 0.105 | |
| 12.58 | | 0.111 | + |
| 1.18 | | 0.103 | |
| 1.40 | | 0.107 | + |
| 2.00 | | 0.109 | |
| 4.30 | | | + |

* Retention catheter in bladder.

leading me to believe that the hepatic artery must play a very important part in carbohydrate metabolism through its blood supply.

IV.

CONCLUSIONS.

1. Saline perfusion of the pancreas directly has no effect upon the blood sugar level and does not cause glycosuria.

2. A glycosuria can be produced by perfusing the liver arteries without an attending marked rise in the blood sugar above a normal level.

3. The vascular supply through the hepatic artery controls a delicate balance in the liberation of sugar from the liver.

The experiments cited are typical of many performed.

I wish to express my deep appreciation to the members of the Physiological Department of Cornell and the X-ray Department of Bellevue Hospital for their kind assistance in this work.

## V.

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# MONOACETONE GALACTOSE.

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Whereas many partially substituted derivatives of glucose are known, thus far few such derivatives have been prepared from galactose. For some investigations now in progress, it became desirable to prepare these. The starting point for such derivatives generally is the monoacetone compound. From galactose this had not yet been prepared.

It was now found that monoacetone galactose is formed in the course of preparation of the diacetone sugar. In contrast to diacetone galactose it has beautiful crystalline properties, which enable its separation from the latter. The details of its purification are given below.

Moncacetone galactose is soluble in water, alcohol, and ethyl acetate. It melts at 157°C. and has a specific rotation of $[\alpha]_D^{20} = -10.9°$. The substance does not reduce Fehling's solution and is not oxidized by the hyperiodite method of Willstätter and Schudel.[1]

## EXPERIMENTAL.

Diacetone galactose was prepared in larger quantities according to the method of Svanberg and Sjöberg.[2] The diacetone galactose was obtained as a thick syrup which distilled at 131–135° at a pressure of 0.2 mm.

In acetylene tetrachloride it had a rotation of

$$[\alpha]_D^{20} = \frac{-2.35° \times 100}{1 \times 4.412} = -53.3°$$

The syrup was allowed to stand at room temperature during the summer months. It was then noticed that crystallization had

[1] Willstätter, R., and Schudel, G., *Ber. chem. Ges.*, 1918, li, 780.

[2] Svanberg, O., and Sjöberg, K., *Ber. chem. Ges.*, 1923, lvi, 863.

started at several points. The syrup was stirred with ether and kept at 10°C. After some months the semisolid contents of the flask were studded with crystalline masses. Further crystallization was hastened by the addition of ligroin and the crystals were filtered off.

After two recrystallizations from ethyl acetate to which ligroin was added, the substance analyzed for monoacetone galactose.

0.1022 gm. substance: 0.1850 gm. $CO_2$ and 0.0700 gm. $H_2O$.

| | | |
|---|---|---|
| $C_9H_{16}O_6$. | Calculated. | C 49.06, H 7.33. |
| | Found. | " 49.36, " 7.65. |

The monoacetone galactose melts sharply at 157°C. and has a specific rotation in ethyl alcohol of

$$[\alpha]_D^{20} = \frac{-0.25^\circ \times 100}{1 \times 2.288} = -10.9^\circ$$

# ON CONDENSATION OF MONOSACCHARIDES BY MEANS OF DILUTE MINERAL ACID.

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The results to be reported in this communication had their origin in an unexpected observation made in the course of the preparation of chitose. It was stated in a previous paper that the 2,5-anhydrosugars are capable of existing in only one form; namely, the aldehydic. Only one 2,5-anhydrohexose has been prepared in crystalline form; namely, epichitose. The preparation of this sugar is very laborious and therefore an attempt was made to obtain it either in a crystalline or at least in a sufficiently pure condition for the preparation of some important derivatives. An unexpected result was obtained at this phase of the work. Instead of an alcohol-soluble product which was expected, a product practically insoluble in all common reagents was obtained. It was insoluble in dilute aqueous alkalies and acids. It could be washed free of adhering mineral acids by means of alkali. On the other hand, the substance was very readily hydrolyzed by heating with very dilute mineral acids. The hydrolysis by boiling with 2 per cent sulfuric acid is completed in approximately 90 minutes. In fact, the substance is 80 per cent hydrolyzed as soon as its solution is accomplished in boiling 2 per cent sulfuric acid. The specific rotation of the completely hydrolyzed and partially hydrolyzed substance is practically unchanged and is the same as that of uncondensed chitose; namely, in the neighborhood of $[\alpha]_D^{25} = +33.0°$ ($\pm 1.0°$). It thus became evident that, under the conditions above described, a condensation of chitose was effected. It is not certain whether the condensation product was a di- or polysaccharide in view of the fact that the insolubility of the product did not permit the measurement of its molecular weight.

An attempt was then made to prepare chondrose. Hope was

entertained that this sugar would be obtained crystalline. Chondrose (2,5-anhydrotalose) has not as yet been prepared. The deamination of chondrosamine was accomplished by means of nitrous acid. The reducing power of the substance was equal to 55 per cent of that of glucose and its specific rotation was found to be $[\alpha]_D^{28} = +20.0°$, calculated on the basis of the quantity of deaminized chondrosamine. When the solution was concentrated under the same condition as that of chitosamine, a solid residue was obtained which was still soluble in water, but only partially soluble in glacial acetic acid. The product which remained insoluble in glacial acetic was no longer soluble in water. When the original substance was redissolved in water and reprecipitated by means of alcohol, the resulting product still possessed some reducing power, but equivalent only to about 22 per cent of the original. On hydrolysis, an increase in reducing power was observed. The specific rotation of the substance was $[\alpha]_D^{28} = 20.5°$. The same value was obtained for uncondensed chondrose. The molecular weight of the soluble product was 351. Thus, it became evident that 2,5-anhydrohexoses condense quite readily. The question naturally arose whether this property was a peculiarity of the aldehydic forms of sugars in contrast with the saccharidic.[1] It therefore became desirable to test the behavior of simple monosaccharides when treated under the conditions which brought about the condensation of the 2,5-anhydrosugars. In the past, glucose has been condensed to di- and polysaccharides, but always through the action of concentrated acids. Experiments here reported deal with glucose, mannose, and lyxose.

*Glucose.*—A solution containing 10 per cent of glucose and 1 per cent hydrochloric acid, evaporated under a pressure of about 10 to 12 mm. in a water bath, temperature of 45–50°C., and further treated as in the experiment of chitose, formed a solid residue which was soluble in water, but insoluble in alcohol and other organic solvents. It was only faintly colored, was no longer sweet, had only a slight reducing power, and a specific rotation of $[\alpha]_D^{20} = +95°$. The reducing power of the substance was about 12 per cent of that of glucose. It was not possible to prepare a phenylosazone of this product. From the hydrolyzed

[1] "Saccharidic" is proposed to be used in place of the confusing terms "glucosidic" or "oxidic."

material, the osazone was readily obtained in very good yield—approximately 1.0 gm. of osazone from 1 gm. of the product. The molecular weight of the substance was 342.

As compared with condensed chitose this substance had the following peculiarities. It was more soluble in water but showed greater resistance towards hydrolytic agents. Apparently it is a disaccharide. The product was completely hydrolyzed by 2 per cent sulfuric acid in a sealed tube at the temperature of the boiling water bath only after 420 minutes, whereas the water-insoluble product of chitose was practically completely hydrolyzed in 90 minutes.

*Mannose and Lyxose.*—These sugars behaved in exactly the same manner as glucose.

In the experiments thus far reported, alcohol was used to bring the product to a state approaching complete dryness and, therefore, there was a possibility that the condensation product was a mixture of the $\alpha$- and $\beta$-ethyl saccharides. True, the solubilities of the condensation products differed from simple ethyl saccharides. Furthermore, it was found that the condensation product was formed even when the step of the operation which involved the use of alcohol was omitted.

As to the structure of the condensation product, little can be said. It may be suggested that in the main, the condensation products are built upon the plan of trehalose. Thus the product formed in the absence of alcohol which was quite impure showed a reducing power equal to 25 per cent of glucose, whereas a disaccharide of the type of maltose should possess the reducing power equal to 50 per cent of that of glucose. However, since the substances are all amorphous, and since the products dried by means of alcohol still contain some alcohol, the possibility is not excluded that admixtures were present of either 1-ethyl disaccharides or of 1-ethyl monosaccharides.

The information obtained from the observations on the reactions with enzymes is not very conclusive. Thus the products were not hydrolyzed by invertase, diastase, and maltase. Emulsin hydrolyzed the condensation products to some extent. Thus, compared with $\beta$-methyglucoside, the condensation product of glucose was hydrolyzed only to the extent varying from 35 to 50 per cent and that of chitose to the extent of 20 to 30 per cent.

XUM

From the view-point of the peculiarities of 2,5-anhydrosugars two very important points were brought out. First, the specific rotations of the condensation products of the simple sugars were higher than those of the equilibrium forms of the sugars whereas those of the 2,5-anhydrosugars had practically the same specific rotations as the original sugars. Such behavior is to be expected in view of the fact that 2,5-anhydrosugars are capable of existing only in the aldehydic form. Second, the condensation products of the 2,5-anhydrosugars seem less stable than those of the simple sugars and this peculiarity also may be attributed to the differences in the functions of carbon atom (1) in the simple and in the 2,5-anhydrosugars.

## EXPERIMENTAL PART.

### *Chitose.*

Chitosamine hydrochloride recrystallized until free from mineral impurities was deaminized in the cold by means of silver nitrite and hydrochloric acid until the amino nitrogen could no longer be detected in the solution. At the end of the operation it was found that 0.1637 gm. of chitose had the reducing power of 0.1000 gm. of glucose. The specific rotation of chitose was found to be $[\alpha]_D^{21} = +33.2°$.

### *Condensation of Chitose.*

The solution obtained on deamination of 20.0 gm. of chitosamine hydrochloride was evaporated in a 1 liter distilling flask under pressure of 11 to 14 mm., from a bath of 40–45°. The remaining syrup was evaporated three times with 75 cc. of 95 per cent alcohol and then four times with 75 cc. of 98 per cent alcohol. The temperature of the bath was allowed to rise to 95–100°. At the final temperature the distillation was continued for 15 minutes. The residue weighed 13 gm. and was a brittle, slightly colored, amorphous substance, insoluble in the usual solvents, glycerol and dilute alkali; dilute mineral acids dissolved it only after hydrolysis. The product was powdered, stirred with 2 per cent ammonia water, filtered, and washed with hot water, alcohol, and ether. The yield was 9 gm. The substance was dried at 60°C. under reduced pressure. The substance turned

faintly yellow at 150°C., and at 222°C. remained solid. The reducing power of the substance suspended in water was equal to 6 per cent of that of glucose. The substance contained about 14 per cent of alcohol when dried at 80°C.

For analysis, the substance was dried at 150°C. under reduced pressure.

0.0956 gm. substance: 0.1760 gm. $CO_2$ and 0.0530 gm. $H_2O$.

| | | |
|---|---|---|
| $C_{12}H_{18}O_9$. | Calculated. | C 47.2, H 5.89. |
| $C_{18}H_{26}O_{13}$. | " | " 48.0, " 5.78. |
| | Found. | " 50.20, " 6.2. |

0.1085 gm. substance: 0.0806 gm. Ag I.

Found. $OC_2H_5$ 14.23.

*Hydrolysis of the Condensed Chitose.*

The reducing power of chitose measured by the Maquenne-Lehman method was found to be equal to 60.7 per cent of that of glucose. Since chitose was available only in solution obtained on deamination of chitosamine, it was desirable to determine the reducing power of crystalline epichitose.

It was found that 0.1000 gm. of glucose had the same reducing power as 0.1640 gm. of epichitose. Hence, the reducing power of epichitose is the same as that found for chitose; namely, 60.7 per cent of that of glucose.

3 gm. of condensed chitose in 150 cc. of 2 per cent sulfuric acid were heated in a sealed test-tube to 100°C. for 30 minutes. The tube was rapidly cooled to 20°C. and the solution distributed into four tubes which were then sealed and heated in a steam bath.

| Time heated. | Reducing power of 0.100 gm. as glucose. | Calculated as chitose. |
|---|---|---|
| *min.* | | |
| 30 | 0.0415 | 0.0681 |
| 60 | 0.0425 | 0.0968 |
| 90 | 0.0493 | 0.0810 |
| 120 | 0.0500 | 0.0820 |
| 150 | 0.0515 | 0.0845 |

The optical rotation was unchanged throughout the experiment and found to be

$$[\alpha]_D^{20} = \frac{+0.64^\circ \times 100}{1 \times 2} = +32^\circ$$

In another experiment, 0.5000 gm. was heated in a steam bath with 25 cc. of 2 per cent sulfuric acid until solution was completed (10 minutes after placing in bath); the solution was rapidly cooled to 25° and the rotation taken in a 1 dm. tube. $[\alpha]_D^{25} = +34.5°$.

The reducing power of 0.1000 gm. is at that time equal to 0.0415 gm. of glucose.

*Condensation of Chondrose.*

When chondrosamine hydrochloride was treated in the same way as glucosamine hydrochloride for the preparation of chitose, a solution was obtained containing 14.5 gm. of chondrose in 250 cc. of solution with a specific rotation of $[\alpha]_D^{22} = +20°$. Its reducing power was equal to 54.64 per cent of that of glucose. The solution, evaporated under the same conditions as chitose, gave a light yellow dry residue which was readily pulverized. The yield was from 9 to 10 gm. from 20 gm. of chondrosamine hydrochloride. The substance had a bitter taste, was partly soluble in glacial acetic acid, and on cooling, partly settled out. This precipitate could not be completely redissolved in water. After reprecipitating a concentrated aqueous solution of the crude product with thirteen times its volume of 98 per cent alcohol, a substance was obtained which had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+0.41° \times 100}{1 \times 2} = +20.5°$$

The reducing power of 0.1000 gm. equals that of 0.0140 gm. of glucose. When once more purified in the same manner, the rotation did not change and the reducing power of 0.1000 gm. was lowered to the value of 0.0125 gm. of glucose. The molecular weight of the substance determined cryoscopically was 351.

*Condensation of Glucose.*

10 gm. of glucose were dissolved in 80 cc. of water. 5 cc. of 10 per cent hydrochloric acid were added and the solution was evaporated under diminished pressure in a 1 liter flask from a bath of 40–45°. The residue was three times evaporated with 75 cc. of 95 per cent alcohol and then four times with 75 cc. of 98 per cent alcohol, allowing the temperature to rise gradually to 95–100° and keeping it at that temperature for 15 minutes. The brittle res-

idue was readily soluble in cold water, reduced Fehling's solution, and had the following optical rotation.

$$[\alpha]_D^{22} = \frac{+1.90° \times 100}{2 \times 1} = +95°$$

0.1000 gm. had a reducing power equal to that of 0.0093 gm. of glucose.

*Rate of Hydrolysis of Condensed Glucose.*—3.0000 gm. of the substance were dissolved in 150 cc. of 2 per cent sulfuric acid and heated in seven separate sealed tubes in a steam bath.

| No. | Time. | Reducing power of 0.1000 gm. equals gm. glucose. | Temperature. |
|---|---|---|---|
| | *min.* | | °C. |
| 1 | 00 | 0.0093 | 95.0 |
| 2 | 30 | 0.0275 | 86.5 |
| 3 | 60 | 0.0376 | 81.0 |
| 4 | 90 | 0.0470 | 78.5 |
| 5 | 120 | 0.0520 | 73.5 |
| 6 | 180 | 0.0655 | 65.5 |
| 7 | 300 | 0.0767 | 65.5 |
| 8 | 420 | 0.0865 | 57.0 |

There remained 74 cc. of the solution containing 1.3 gm. of glucose. The free sulfuric acid was neutralized with sodium acetate and to this solution 2.2 cc. of phenylhydrazine and 2 cc. of glacial acetic acid were added and the solution was allowed to stand on the water bath for 1½ hours. The yield of glucophenylosazone was 1 gm. Once recrystallized from methyl alcohol, it melted at 204–205°. The osazone in pyridine-alcohol (2:1) at 21° in a 1 dm. tube gave $\alpha_D = -0.72°$ ($c = 1$). After 24 hours: $\alpha_D = -0.28°$.

$C_{18}H_{22}O_4N_4$. Calculated. N 15.47.
Found. " 15.43.

Molecular weight determination by the cryoscopic method gave a value of 309.

*Condensation of Mannose.*

10 gm. of mannose with the specific rotation of $[\alpha]_D^{22} = +14.5°$ (having the reducing power of 0.095 gm. of glucose) were dissolved in 125 cc. of water and 10 cc. of 10 per cent hydrochloric acid.

XUM

This solution was treated in the same manner as the solution of glucose. The yield was 10 gm. of a bitter tasting, brittle, amorphous substance, soluble in water. It was redissolved in 10 cc. of warm water and precipitated with 240 cc. of absolute alcohol. The operation was repeated twice and the final yield was 6.5 gm. The molecular weight was 378.

The optical rotation of the substance was as follows:

$$[\alpha]_D^{28} = \frac{+1.19° \times 100}{1 \times 2} = +59.5°$$

The substance did not reduce Fehling's solution. The reducing power of the substance after hydrolysis for 16 hours in a sealed tube placed in a steam bath was equal to 80 per cent of its weight of glucose and the specific rotation of the product of hydrolysis calculated on this basis was $[\alpha]_D^{20} = +13°$.

From this solution a levorotatory phenylosazone was obtained which had a melting point of 203–205°C. and had the following composition.

0.1000 gm. substance: 13.7 cc. nitrogen gas at 26°C. and 757.4 mm.

$C_{18}H_{22}O_4N_4$. Calculated. N 15.47.
Found. " 15.59.

*Condensation of Mannose in the Absence of Alcohol.*

10 gm. of mannose were dissolved in 125 cc. of water and 10 cc. of 10 per cent hydrochloric acid. This solution was evaporated under reduced pressure from a bath of 45°C. (1 liter flask, wide capillary, pump of large capacity). After 20 minutes the contents of the flask had a syrupy consistency. The temperature of the bath was slowly raised to 65° when the syrup began to solidify. 10 minutes later the temperature was raised to 73°. The reaction was interrupted and the solid, somewhat colored product was removed from the flask. It still contained a little hydrochloric acid. The yield was about 8 gm.

The optical rotation of the substance was

$$[\alpha]_D^{21} = \frac{+0.33° \times 100}{1 \times 1} = +33°$$

The substance was dissolved in 5 cc. of water and reprecipitated with absolute alcohol. The optical rotation of the substance then rose slightly to

$$[\alpha]_D^{25} = \frac{+0.74^\circ \times 100}{1 \times 2} = +37^\circ$$

The reducing power of this substance was equal to 20.5 per cent of its weight of glucose. The reducing power rose to 82.5 per cent of glucose after an 8 per cent solution of the substance in 2 per cent sulfuric acid was heated for 6 hours in a sealed tube placed in a steam bath. The specific rotation of the mannose in the final solution was $[\alpha]_D^{25} = +13.75^\circ$.

*Condensation of Lyxose.*

10 gm. of lyxose ($[\alpha]_D = -13^\circ$) were dissolved in 90 cc. of water and 10 cc. of hydrochloric acid and the solution was concentrated in the same manner as described in previous experiments. The yield was 10.0 gm. of a dry substance easily pulverized and very soluble in water. The molecular weight of the substance was found to be 338.

The optical rotation of the substance was the following.

$$[\alpha]_D^{25} = \frac{+0.83^\circ \times 100}{1 \times 2} = +41.5^\circ$$

The substance had the following composition.

0.1020 gm. substance: 0.1728 gm. $CO_2$ and 0.0618 gm. $H_2O$.
0.0975 " " : 0.0538 " AgI.

The composition of the substance approached that of a dipentose with 1 mol of alcohol.

| | | | | |
|---|---|---|---|---|
| $C_{10}H_{18}O_9 + C_2H_5OH$. | Calculated. | C 43.9, | H 7.3, | $OC_2H_5$ 13.7. |
| | Found. | " 45.75, | " 6.71, | " 10.57. |

The substance was hydrolyzed by means of a 2 per cent solution of sulfuric acid. After 14 hours the reducing power of the hydrolysate was equivalent to 85 per cent of glucose. From the hydrolysate a phenylosazone was obtained which melted at 162°C., was levorotatory, and had the following composition.

0.0965 gm. substance: 15.00 cc. nitrogen gas at 24° C. and 749.4. mm.

| | | |
|---|---|---|
| $C_{17}H_{20}O_3N_4$. | Calculated. | N 17.07. |
| | Found. | " 17.60. |

XUM

# FURTHER STUDIES ON THE PHYSIOLOGICAL ACTION OF A PARATHYROID HORMONE.

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## INTRODUCTION.

In a previous communication (1) it has been shown that, by weak acid hydrolysis of the fresh parathyroid glands of the ox, an extract could be obtained which contained an active principle. It has been found that by the use of this active principle complete replacement therapy was afforded in the case of parathyroidectomized dogs. The results of a number of preliminary experiments, in which the physiological effects were noted following the administration to normal dogs of extracts containing this active principle, have also been reported (2). In this communication there are reported a number of experiments which represent for the most part an extension of the previous study above referred to. Over 250 animal experiments have been carried out and results have been in remarkable agreement.

### *Methods.*

The extract used in this series of experiments was a more pure and stable product than that used in the earlier work. Also it was made up in relatively large amounts so that quantitative experiments could be made. After the preliminary investigations which proved the efficacy of our preparation had been carried out, an attempt was made to purify further and stabilize the active principle. It appeared to be a substance of the nature of a complex protein derivative or else a substance intimately associated with such type of compound. It was therefore possible to separate the active principle from the original extract by salt-



XUM

ing-out processes. Such precipitates were purified further by dialysis and by isoelectric fractionation. The chemical study of our extracts will be reported upon separately.

Both normal and thyroparathyroidectomized dogs were used in this study. Blood samples for chemical analysis were taken as required from a leg vein. The extracts were administered by subcutaneous injection. Calcium was determined on blood serum only. The method used was the Clark-Collip modification of the Tisdall technique (3). Chlorides were determined on whole blood by the method of Van Slyke (4). Phosphorus and magnesium were determined by the Briggs method (5). During the experiments animals were fed meat, and stock animals were fed on kitchen scraps containing considerable meat.

## RESULTS.

For purposes of convenience the results of many typical experiments are shown in the form of graphs.

### *Overdosage Phenomena.*

Injections of even very small amounts of potent extract repeated at intervals of a few hours into either normal or thyroparathyroidectomized animals have invariably proven fatal when the injections were continued. Such results were due to long maintained hypercalcemia induced in the first place by the pyramiding of the effect of each successive injection upon the preceding one. As a general rule hypercalcemia was without ill effect upon normal or thyroparathyroidectomized dogs if the condition were not maintained. In other words, the production of a condition of hypercalcemia (values over 15 mg.) by repeated injections of extract caused no ill effects in the vast majority of cases if injections were discontinued immediately after the high point in blood serum calcium was attained. The first definite symptom of parathyroid hormone overdosage was vomiting. Cessation of injections, when vomiting was first noted, led, as a rule, to no fatal issue. Blood serum calcium values as high as 23 mg. have been observed in cases where spontaneous recovery has occurred.

In sharp contrast to the lethal effect on dogs of repeated in-

jections of the hormone, was the comparatively harmless action of even massive single doses of the same extract. It was found that a single injection of our parathyroid preparation produced,

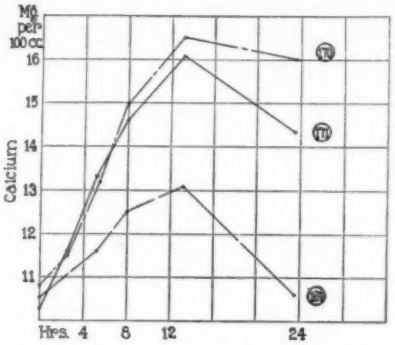


CHART 1. Dog 169, 22 kilos, 10 cc. of parathyroid extract.
" 171, 20 " 15 " " " "
" 170, 19 " 20 " " " "

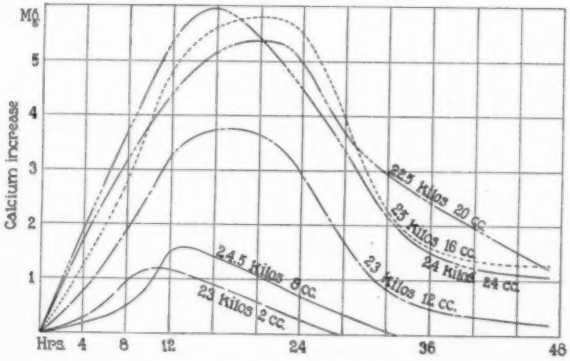


CHART 2. Constructed curves showing effect of increasing dosage (simple injections) upon blood serum calcium values of normal dogs.

as a rule, no untoward symptoms in normal dogs. It was also found that the blood serum calcium rose to a certain peak value within some hours following a single injection and then gradually returned to within normal limits. The calcium-raising effect

of a single injection appeared to stand in direct relationship to the size of the dose administered (Charts 1, 2, 3, and 4). It was only rarely that normal animals, submitted to massive single injections of potent extract, were observed to vomit during the period of hypercalcemia which ensued. When vomiting did occur no other ill effect was observed and such animals were otherwise

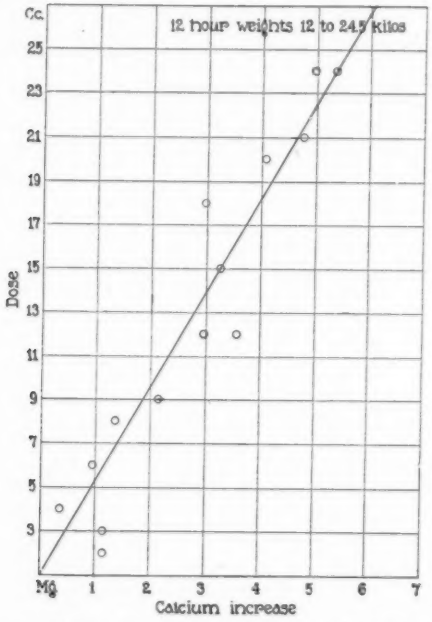


CHART 3. The circles indicate blood serum calcium increase over 12 hours following a single injection of extract into normal dogs plotted against dosage. The same extract was used throughout.

normal. Blood serum calcium values as high as 18 mg. have been observed following a single injection. A unit of potency has been provisionally defined as 1/100 of the amount of extract which will produce an average increase of 5 mg. in the blood serum calcium of normal dogs of approximately 20 kilos weight over a period of 15 hours.

The phenomena of overdosage produced by repeated injec-

tions of potent extract into both normal and thyroparathyroidectomized dogs have been studied quite extensively. In these experiments animals were injected at 4 hour intervals with approximately 25 units of parathyroid hormone. The injections were continued irrespective of the condition of the animal until

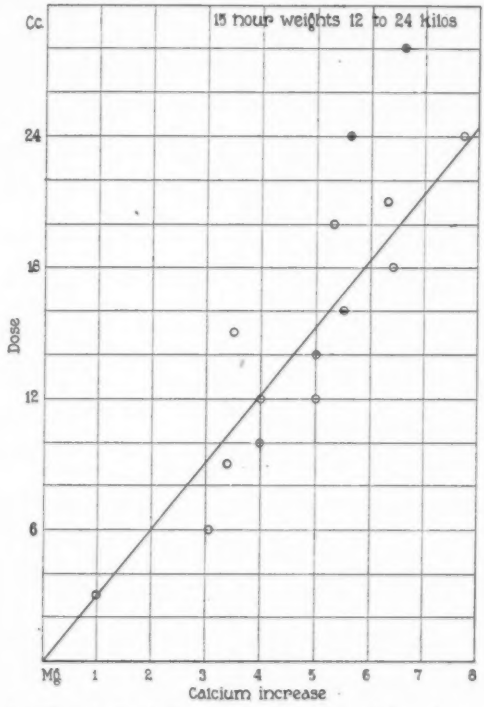


CHART 4. The circles indicate blood serum calcium increase over 15 hours following a single injection of extract into normal dogs. The same extract was used throughout.

death ensued. Blood samples for analysis were taken at frequent intervals. Charts 5, 6, 7, 8, and 9 illustrate the results of five experiments carried out simultaneously. Dogs 175, 176, and 177 were normal animals, while Dogs 204 and 206 were thyroparathyroidectomized. Another thyroparathyroidectomized

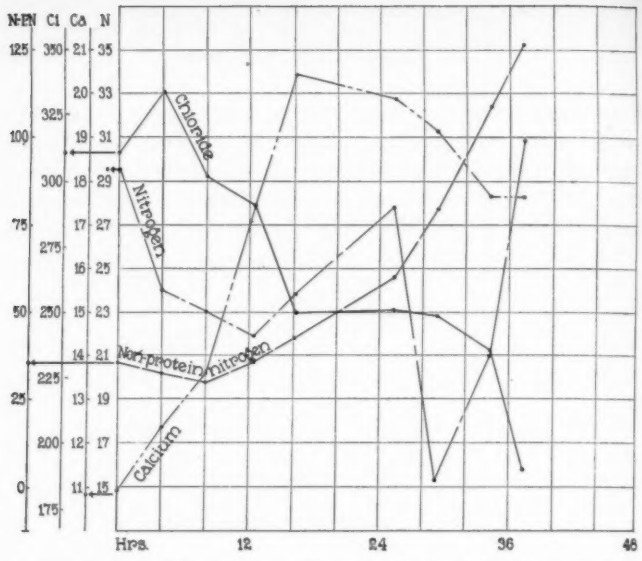


CHART 5. Dog 175, 21 kilos. Normal animal. Injected every 4 hours with 5 cc. of extract.

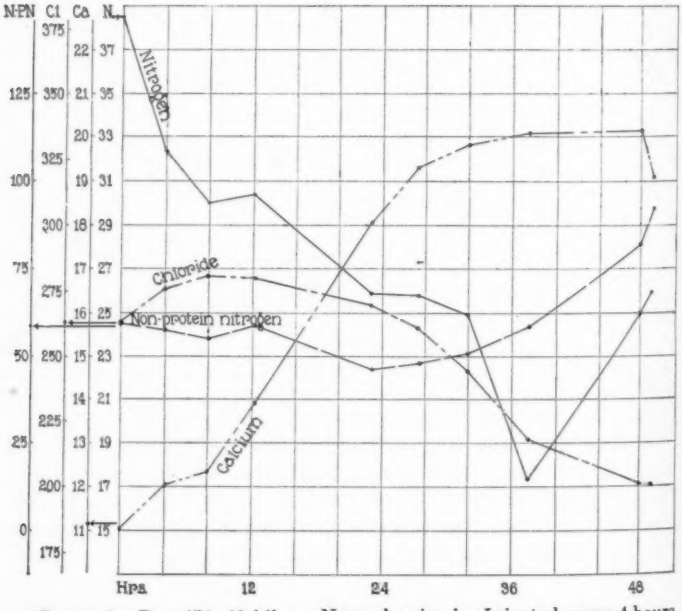


CHART 6. Dog 176, 19 kilos. Normal animal. Injected every 4 hours with 5 cc. of extract.

animal was also run in this series. This animal, Dog 207, had considerable hemorrhage from the neck wound during the experiment. It died 13 hours after the beginning of the experiment and the blood serum calcium at death was 12.6 mg. while the control value was 7 mg. Thyroparathyroidectomy had been performed on Dogs 204, 206, and 207, 5 days previous to this experiment. They had also been treated with extract to

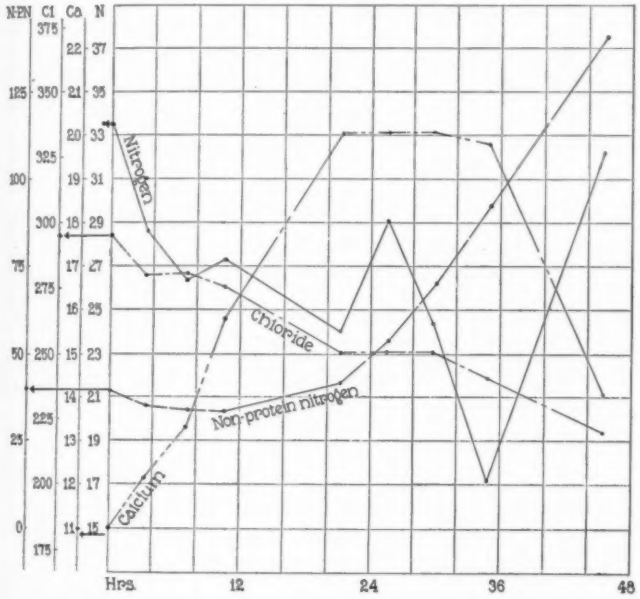


CHART 7. Dog 177, 19.5 kilos. Normal animal. Injected every 4 hours with 5 cc. of extract.

offset the development of tetany. Dog 204 was moribund after 4 days and an attempt was made at this point to resuscitate this animal by intravenous saline injection. The animal died, however, 2 hours later without having manifested any improvement. The last blood analyses shown on Chart 8 were made on the sample taken prior to the intravenous saline injection and are not terminal figures in this instance. At death the blood serum

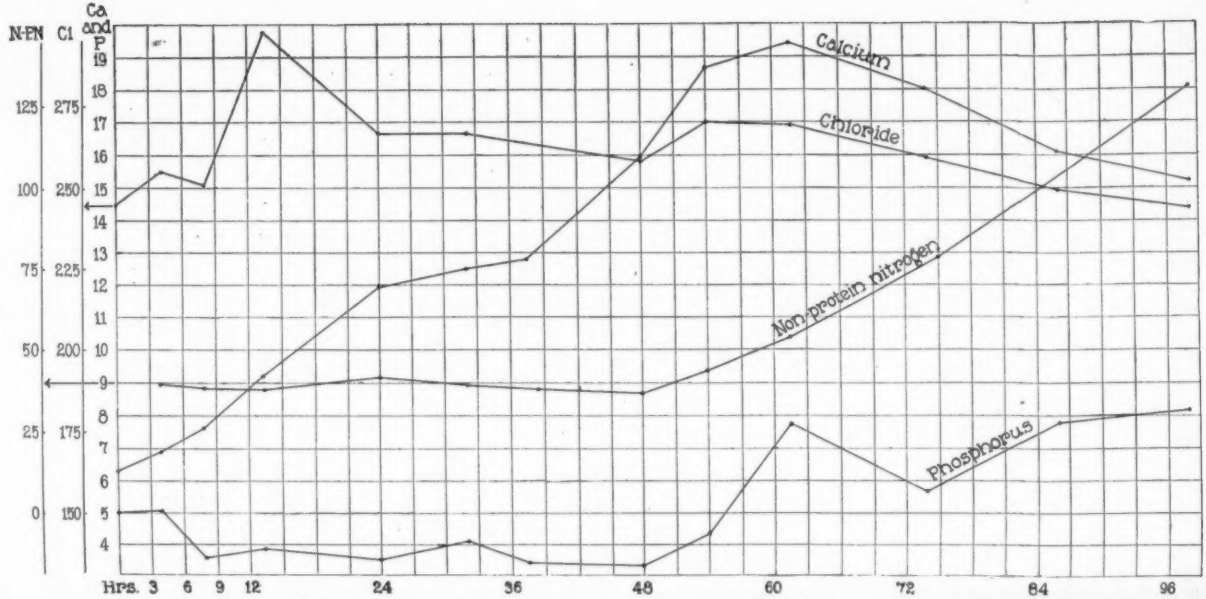


CHART 8. Dog 204, 32 kilos. Thyroparathyroidectomized animal. Injected every 4 hours with 5 cc. of extract.

CHART 8. Dog 204, 32 kilos. Thyroparathyroidectomized animal. Injected every 4 hours with 5 cc. of extract.

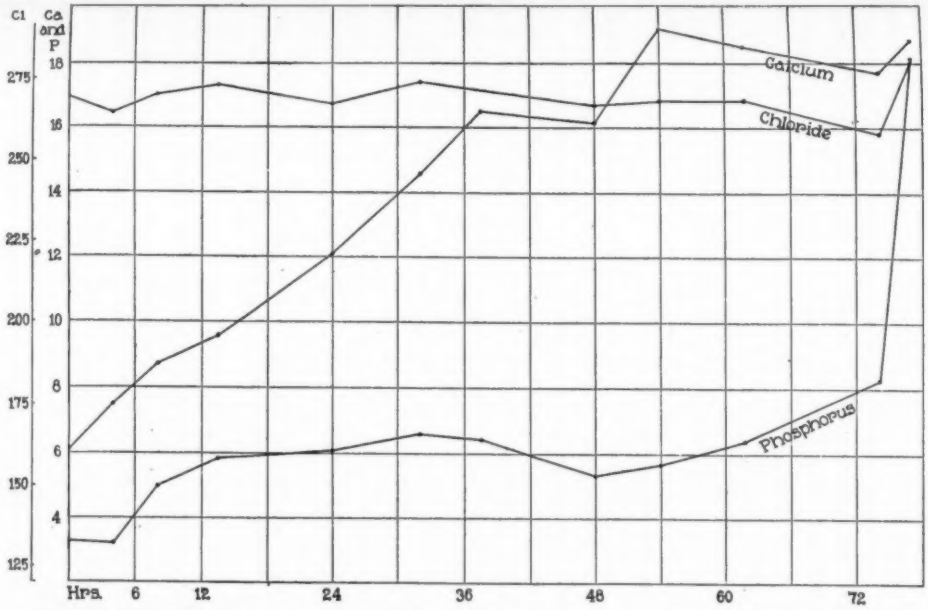


CHART 9. Dog 206, 29 kilos. Thyroparathyroidectomized animal. Injected every 4 hours with 5 cc. of extract.

calcium was 11.7 mg. and the whole blood phosphorus, 8 mg. 1,500 cc. of normal saline solution had been injected.

No satisfactory antidote which will offset the fatal issue in such experiments as those outlined above has as yet been found. The calcium content of the blood serum has been reduced by various measures. After a period of many hours of extreme hypercalcemia, death has invariably occurred in our experience. As has been pointed out, however, hypercalcemia of short duration appears to be harmless. For this reason it is very hard to determine definitely the effectiveness of possible antidotes since short periods of hypercalcemia have been found to be harmless and long ones fatal, and in those of intermediate duration, spontaneous recovery occurred as a rule.

In order to determine the possible beneficial effect of hypertonic saline or glucose solutions, a number of experiments have been carried out in which, in addition to the administration of approximately 25 units of parathyroid hormone at 4 hour intervals, intravenous injections of one or other of 15 per cent NaCl or 25 per cent glucose solution were made twice daily. By such measures it was shown that the fatal issue could be offset by many hours or even days. Chart 20 illustrates the blood calcium curve in a dog which lived for 7 days. Injections of the hormone were continued for 5 days while 150 to 200 cc. of 25 per cent glucose were administered twice daily from the 2nd to the 6th day.

*Comparison between the Effects of the Hormone upon Normal and Thyroparathyroidectomized Dogs.*

In our earlier experiments overdosage phenomena ending fatally were encountered in a number of thyroparathyroidectomized animals receiving two injections per day. As no ill effect had at that time ever been observed in normal dogs receiving two injections in a single day, it appeared to us that the normal dog was less sensitive to the hormone than the operated animal. Also it had been observed at that time that tetany might be relieved in a thyroparathyroidectomized animal by an extract which had in a single dose little or no effect on a normal animal. An attempt was therefore made to make a rigid comparison between the effect of equal doses of a standard extract on normal and

thyroparathyroidectomized dogs of the same weight. Charts 5, 6, 7, 8, and 9 are illustrative of one type of such comparative experiments, while Charts 10, 11, and 12 demonstrate another method of

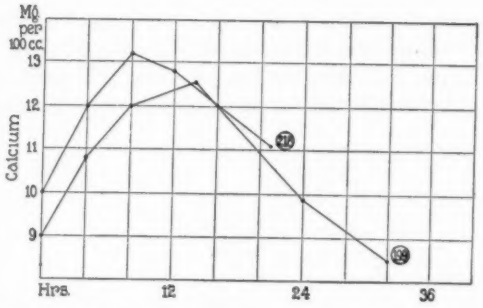


CHART 10. Dog 218, 15 kilos. Normal animal. Injected with 5 cc. of extract.

Dog 199, 19 kilos. Thyroparathyroidectomized animal. Injected with 5 cc. of extract.

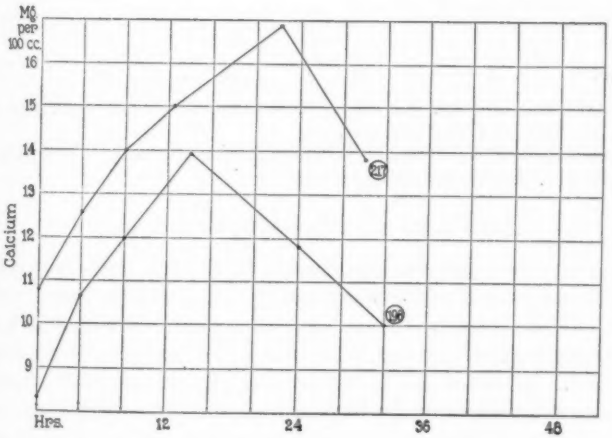


CHART 11. Dog 217, 18.75 kilos. Normal animal. Two injections of 5 cc. of extract at a 4 hour interval.

Dog 196, 19.5 kilos. Thyroparathyroidectomized animal. Two injections of 5 cc. of extract at a 4 hour interval.

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such comparison. In addition to these experiments four normal dogs were injected with varying doses of the same extract, the injections being made at 12 hour intervals. The smallest dose used was 1 cc. and the largest 4 cc. The animal receiving 3 cc. of extract died on the 3rd day; the animal receiving 4 cc. died on the 4th day; the animal receiving 1 cc. died on the 6th day: and the animal receiving 2 cc. died on the 8th day. All showed typical symptoms of hypercalcemia for many hours before death occurred.

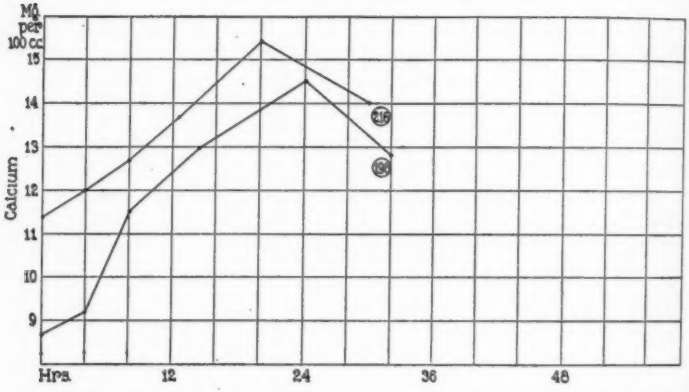


CHART 12. Dog 216, 18.5 kilos. Normal animal. Three injections of 5 cc. of extract at 4 hour intervals.

Dog 198, 19 kilos. Thyroparathyroidectomized animal. Three injections of 5 cc. of extract at 4 hour intervals.

### *Blood Volume as Influenced by the Hormone.*

In order to circumvent the effect of bleeding, a series of experiments was carried out in which normal dogs were injected with 5 cc. of extract every 4 hours until they died. Hemoglobin was determined at frequent intervals by a macro-acid-hematin method. As there can be no appreciable change in the total hemoglobin content of the blood during such an experiment, any fluctuations in hemoglobin readings must be due to dilution or concentration of the blood. Setting the colorimeter at 10 for the tube containing the first blood sample and using this as a control, the colorimeter readings for the succeeding samples have been plotted against time (Chart 13).

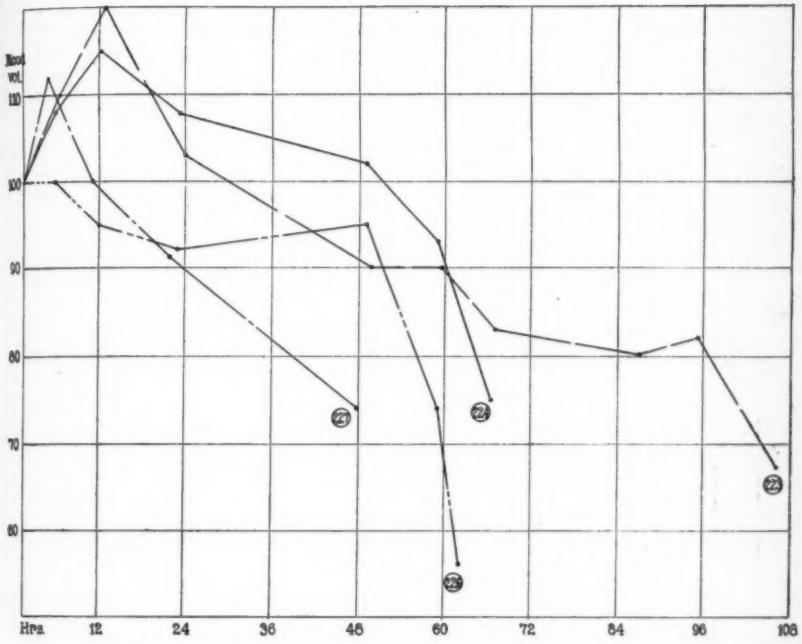


CHART 13. Four normal dogs injected every 4 hours with 5 cc. of extract.

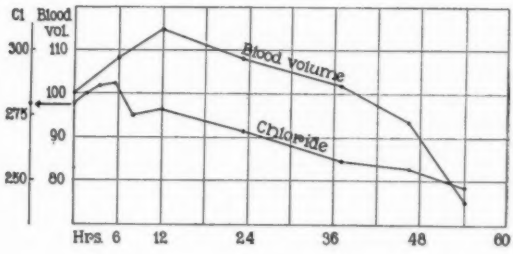


CHART 14. Dog 224. Normal dog injected every 4 hours with 5 cc. of extract.

Charts 14 and 15 illustrate the fluctuation in blood volume (hemoglobin method) and total chlorides as induced by overdosage with the parathyroid extract.

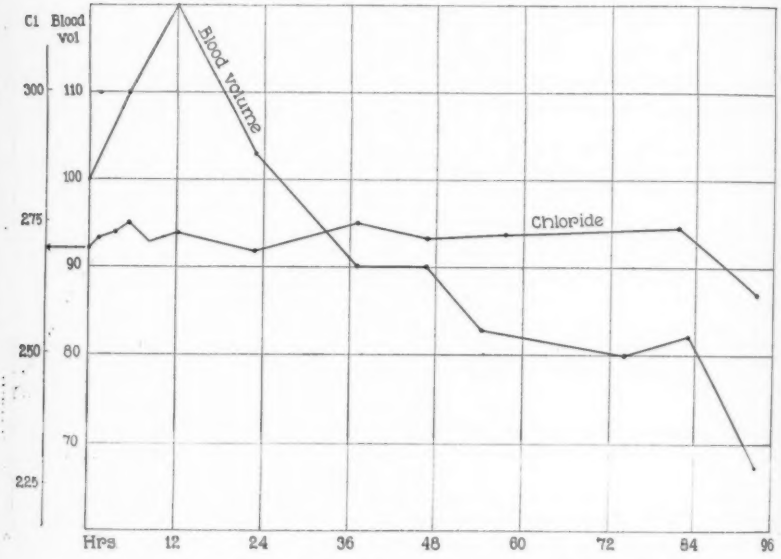


CHART 15. Dog 223. Normal dog injected every 4 hours with 5 cc. of extract.

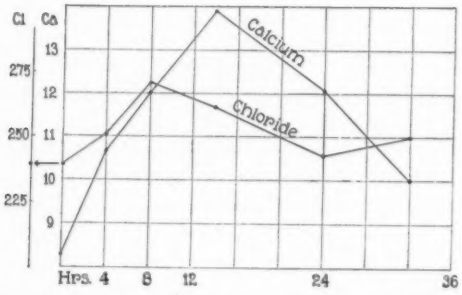


CHART 16. Dog 196. Thyroparathyroidectomized dog. Two injections of 5 cc. of extract at a 4 hour interval.

In Charts 16, 17, and 18 are shown the blood serum calcium and the whole blood chlorides in thyroparathyroidectomized dogs injected with parathyroid extract.

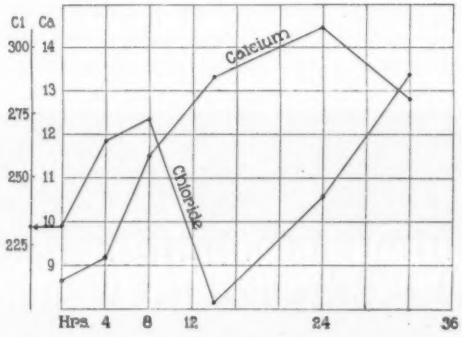


CHART 17. Dog 198. Thyroparathyroidectomized dog. Three injections of 5 cc. of extract at 4 hour intervals.

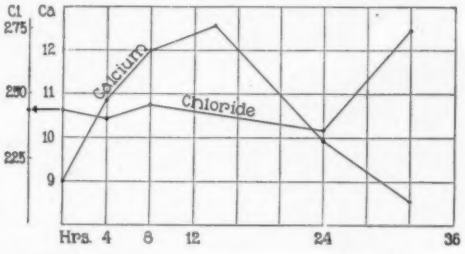


CHART 18. Dog 199. Thyroparathyroidectomized dog. One injection of 5 cc. of extract.

*Effect on Blood Pressure.*

Chart 19 illustrates the effect of injection of the parathyroid extract upon the blood pressure.

*Elevation of Blood Serum Calcium in Anesthetized Animals.*

It has been found possible to elevate the blood serum calcium in normal dogs under amytal anesthesia. In one experiment a

5 mg. rise was produced over 14 hours and in another experiment a 3 mg. rise was noted over 3 hours.

*Relationship of the Hormone to Guanidine.*

Guanidine has been injected along with the parathyroid extract into three normal dogs. The injections have been repeated until death resulted. One control animal received guanidine

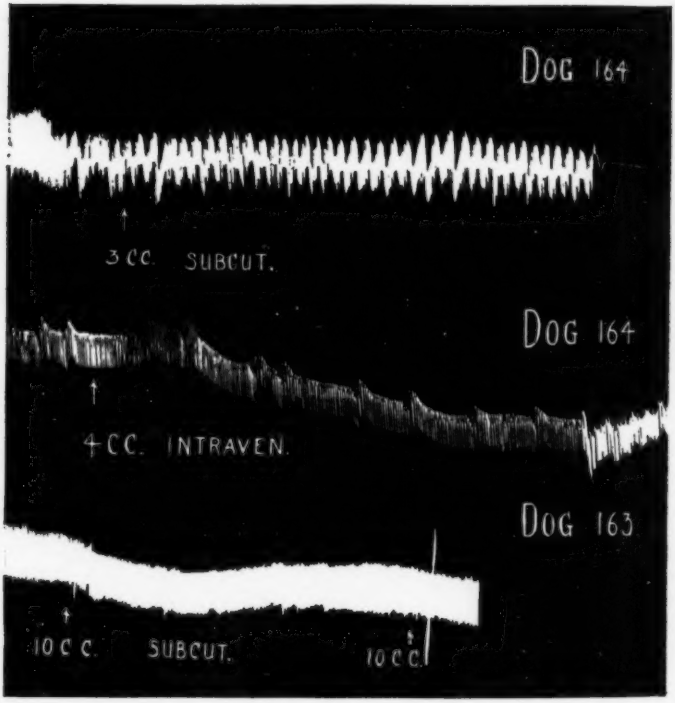


CHART 19. Showing effect of injection of extract upon blood pressure in anesthetized animals. Middle tracing fast drum.

alone. The extract used was a standardized preparation and had been repeatedly used on other normal dogs. All the animals developed typical tetany of a very severe type on the 2nd day.

All died shortly thereafter. The blood serum calcium in the three animals receiving parathyroid extract in addition to guanidine was, just prior to death, when the animals were in a state of tetany, respectively 17.8, 18.4, and 19.1 mg. per 100 cc. The

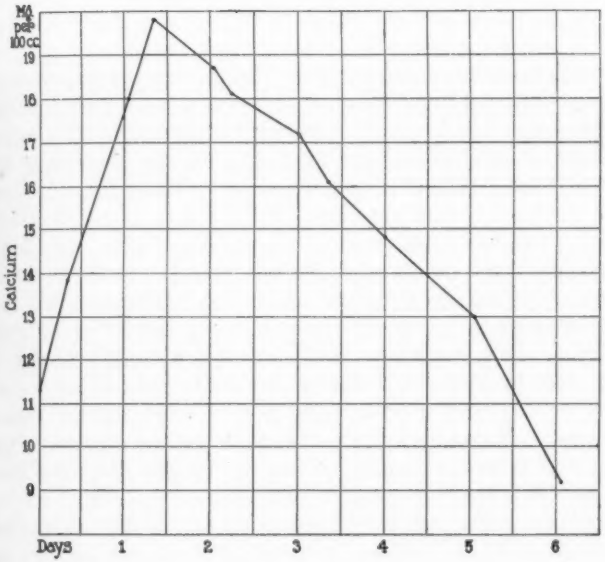


CHART 20. Dog 274, 27 kilos. This animal was injected at approximately 4 hour intervals with 25 units of parathyroid extract. The injections were continued for 5 days. Starting on the 2nd day 150 to 200 cc. of 25 per cent glucose were injected twice daily until the 6th day. The animal died on the 7th day. There was hematemesis during the last 48 hours of the experiment.

control animal receiving guanidine alone showed an increase of 2.5 mg. of calcium over the period of the experiment. No antagonistic influence of guanidine upon the hormone was evident in these experiments. It is possible, however, that such antagonism may be demonstrated by other means.

DISCUSSION.

The results herein reported taken in conjunction with the previous experiments indicate that a condition of profound hypercalcemia induced in dogs by overdosage through repeated injections with this hormone invariably ends in death. The relatively harmless action of single massive doses of the hormone is by contrast a matter of great interest and also of much practical importance. The discovery that the blood serum calcium-raising effect of a single injection of a potent parathyroid extract was directly related to the size of the dose administered, afforded a means of physiological standardization of the potency of any given extract. It is, of course, necessary, as the results of several experiments indicated graphically in Charts 1, 2, and 3 will illustrate, to use several animals in a potency test. In all the experiments, the results of which are indicated in these charts, the same extract was used throughout. It should be noted that there was considerable variation in the response of different animals. This variation could not be entirely related to differences in weight and dosage for it has been found on occasion that a large animal might show a greater effect than a small one. It has been found that the best way to make a potency test is to use several animals of approximately the same weight. If it is not convenient to determine the actual calcium curve in its entirety, it would appear that a reliable comparison of effects could be obtained by noting the calcium increase resulting over a definite period such as 12 to 15 hours. It is suggested that the approximate dosage used should be varied from 50 to 200 units as previously defined. One cannot emphasize too much the importance of using many dogs in potency testing. It has been our experience that one lot of animals may give results which are in remarkable agreement with the suggested standardization curves shown in Charts 2 and 3, while another lot may show only partial agreement, with one or two animals showing calcium increases which are far removed from the theoretical values. These results may be due to inadequate control of the diet in the preexperimental period in these cases. It is felt, however, that all such divergent readings will be compensated for if sufficient animals are used. It is also recommended that

animals used in potency testing be given a rest of some days between successive tests.

It is also recommended that animals used in potency testing be kept on a standard diet both before and during the actual experimental period. Due to the fact that we have observed that the rabbit is relatively immune to repeated injections of potent parathyroid extract, whereas the dog, as the foregoing experiments have demonstrated, has been shown by contrast to be highly sensitive to this extract, we were led to suspect that the factor of diet might influence to a considerable extent the blood serum calcium curve of dogs treated with the hormone. To test out this hypothesis, two groups of four dogs each were

TABLE I.

| Dog No. | Weight. | Diet. | Amount of extract given. | Blood serum calcium. | | Ca increase. |
|---|---|---|---|---|---|---|
| | | | | Control. | After 15 hrs. | |
| | *kg.* | | *cc.* | *mg.* | *mg.* | *mg.* |
| 284 | 23 | Bread. | 10 | 11.0 | 13.0 | 2.0 |
| 285 | 20.5 | " | 15 | 10.6 | 14.2 | 3.6 |
| 286 | 17 | " | 20 | 11.1 | 15.9 | 4.6 |
| 287 | 15 | " | 25 | 11.1 | 14.7 | 3.6 |
| 288 | 16 | Meat. | 10 | 11.1 | 14.6 | 3.5 |
| 290 | 17 | " | 15 | 11.7 | 12.7 | 1.0 |
| 289 | 24 | " | 20 | 11.1 | 14.1 | 3.0 |
| 291 | 20 | " | 25 | 10.6 | 13.7 | 3.1 |

kept respectively on an exclusive lean meat diet and an exclusive bread diet for a period 1 week prior to a potency test. These eight animals then received single injections of varying amounts of a standard extract. The results are shown in Table I.

From the results of Table I one might conclude that dogs kept on a bread diet react more uniformly to the extract than do those which are meat-fed. Our previous experience, however, has been that meat-fed dogs also react with a fair degree of uniformity to the extract as Charts 1, 2, 3, and 4 illustrate.

The experiments in which certain changes in the composition of the blood induced by overdosage with the hormone have been followed are of considerable interest. Charts 5, 6, 7, 8, and 9 illustrate the curves for calcium, chlorides, total nitrogen, and

non-protein nitrogen in both normal and parathyroidectomized dogs submitted to overdosage with potent extract. Inorganic phosphorus has also been determined in certain instances. These results must be discounted by the factor of bleeding. The amount of blood withdrawn from each animal during the course of the experiment was considerable and this fact must have influenced to a greater or less degree the results obtained. The gradual fall in total nitrogen during the major part of any experiment, for example, is probably due in the main to bleeding. The preterminal rise in total nitrogen is on the contrary directly related to the action of the hormone injected. In a similar manner one might attribute the gradual fall in total chlorides to the bleeding.

In those instances where the effect of bleeding was practically excluded it was found that the chloride curve was not typical. Frequently a definite decrease in blood chlorides was noted terminally and there was also noted a slight increase in the early hours of some experiments.

The blood phosphorus showed very little fluctuation during the period of calcium increase. However, with the establishment of marked hypercalcemia the phosphorus increased progressively, and in the last few hours of life the phosphorus values actually soared to very high levels.

The curves for non-protein nitrogen show quite conclusively that the great increase in this nitrogen fraction is a preterminal phenomenon and that it should in no wise be associated with the early and more typical physiological action of the hormone; namely a blood calcium-mobilizing effect.

The calcium curves were the most typical of the action of the hormone. They took the following general form: A gradual ascent to a peak point in the neighborhood of 20 mg. per 100 cc., a plateau effect at this level which was followed, in spite of the continuation of the injections, by a gradual fall to lower levels, from which in some instances it rose again just prior to death. It would appear that there is a definite limit to the degree of calcium mobilization possible in the blood. This upper limit is somewhere in the neighborhood of 20 mg. per 100 cc. After this concentration of calcium was attained further injections of potent parathyroid extract could only act to maintain this level

and, as the results often showed, an actual fall in calcium value might be observed in the latter stages of such experiments. The fall in calcium at this time was associated with, if not directly due to, the mobilization of phosphorus. No changes in blood serum magnesium content which were beyond the limit of experimental error have been observed in experimental hypercalcemia.

A very definite attempt was made to discover by quantitative experiments if the thyroparathyroidectomized dog was any more sensitive to the extracts being studied than was the normal animal. The data set forth in Charts 5 to 12 inclusive show quite conclusively that, in as far as the calcium-raising effect of the hormone is concerned, there was little or no difference between the response to injections of the extract to normal and thyroparathyroidectomized dogs.

The fluctuations in hemoglobin content of the blood of normal dogs subjected to overdosage with the hormone would indicate that the preliminary effect of the injections is to cause a slight dilution of the blood, while in the terminal stages the marked increases in hemoglobin content would indicate a great decrease in the plasma volume.

In uniformity with most other tissue extracts, the intravenous injection of our purified extracts causes a slight fall in blood pressure which, however, quickly regains its normal level. Subcutaneous injections of the same extracts, unless very massive doses are used, have little or no immediate effect on blood pressure.

The demonstration that mild hypercalcemia can be induced in a fully anesthetized animal by the use of the parathyroid hormone is of interest.

It is not our intention to make any definite pronouncement at this time on the relation of guanidine to the physiological action of the parathyroid hormone being studied. We have been able to produce tetany in dogs by guanidine and at the same time have observed the typical overdosage effects of our extract. It is quite possible that there is an antagonism or an interrelationship between the parathyroid glands and guanidine compounds. Our experiments so far, however, have failed to disclose any such condition.

We now have one animal which was thyroparathyroidectomized

XUM

some 5 months ago. This animal has manifested violent tetany up to this date at any time that the daily dose of extract has been omitted. The animal is in perfect condition and has been on an exclusive meat diet during the whole period. Ample evidence has therefore been obtained to indicate that the parathyroid extract which is being used in this laboratory contains a substance or substances which will afford complete replacement therapy in the case of the totally parathyroidectomized dog.

The methods which we have developed for testing extracts of parathyroid glands for potency have thus far shown that any extract of the fresh ox glands, which is sufficiently concentrated and more or less potent and which has been made by a process of weak acid hydrolysis, contains more or less of the active principle. In view of these results it would seem fair to conclude that all previous investigators in this field who have had or used at one time or another fresh extracts made by weak acid hydrolysis of fresh ox glands must have had more or less of the active principle in their preparations. Chief among such workers in recent years who have prepared and used acid extracts of parathyroid glands is Hanson (6). This author's papers have only recently come to the attention of the present writers. A careful perusal of them indicates that Hanson undoubtedly must have had some of the active principle in certain of his extracts. The contribution which Hanson has made in this field we are glad to recognize and direct attention to.

## SUMMARY.

1. The preliminary experiments on the physiological action of a hormone contained in certain parathyroid gland extracts have been extended and amplified.

2. The active principle has been obtained in a more purified, concentrated, and stable form.

3. A method of physiological standardization of the potency of extracts of parathyroid glands has been suggested.

4. A provisional unit of potency has been defined.

5. Overdosage phenomena have been studied in detail.

6. Simultaneous administration of potent parathyroid extract and guanidine to normal dogs at regular intervals has resulted in a condition of tetany and of profound hypercalcemia. Death

has resulted in such experiments within approximately the same time as in the case of animals receiving parathyroid extract only.

7. The calcium-raising effect of the hormone is practically the same in the thyroparathyroidectomized dog as in the normal animal.

It is a pleasure to acknowledge the financial assistance afforded for this research by a grant from the Carnegie Foundation.

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XUM

# STUDIES IN CALCIFICATION.*

## I. THE SOLUBILITY PRODUCT OF SECONDARY AND TERTIARY CALCIUM PHOSPHATE UNDER VARIOUS CONDITIONS.

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CONTENTS.

| | PAGE |
|---|---|
| I. Introduction | 509 |
| II. Titration curves of phosphoric acid | 511 |
| III. The solid phase at equilibrium | 516 |
| IV. Calculation of the $PO_4^{\equiv}$ ion concentration | 518 |
| V. Experimental technique | 522 |
| VI. Solubility product of $Ca_3(PO_4)_2$ | 523 |
| VII. Complete dissociation and the theory of neutral salt effects | 526 |
| VIII. Effect of valency and the concept of ionic strength | 531 |
| IX. Solubility product and activity coefficient of $Ca_3(PO_4)_2$ in salt solutions | 534 |
| X. Solubility product of $Ca_3(PO_4)_2$ in blood serum | 546 |
| XI. Solubility product of $CaHPO_4$ in salt solutions | 549 |
| XII. Calcium buffers | 552 |
| XIII. Solubility relations of phosphates in the animal organism | 561 |
| XIV. Summary | 564 |

### I.

### INTRODUCTION.

The problems of bone calcification, pathological calcification, and the absorption and excretion of calcium and phosphorus

* An abstract of the three papers of this series was published in the Proceedings of the Society for Experimental Biology and Medicine (Holt, L. E., Jr., La Mer, V. K., and Chown, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1924-25, xxii, 283).

† A part of the experimental work described in this paper was carried on in the Marine Laboratory of The Rockefeller Institute, Woods Hole, Mass. We wish to thank Dr. Simon Flexner for his kindness in allowing us to use these facilities.



XUM

from the body are intimately connected with the solubllities of the sparingly soluble salts of calcium, the most important of which is the tertiary phosphate $Ca_3(PO_4)_2$. Without an exact knowledge of the solubility of this salt, quantitative studies of the equilibria in which it is concerned are out of the question.

In his recent discussion of this problem L. Michaelis (1) has made the following pertinent remarks:

"An diesem Beispiel sieht man, wie zur klaren Erkenntnis physiologischer Dinge oft die exakte Kenntnis von Naturkonstanten wichtiger ist als Hypothesen. Es ist zu verwundern, dass derartige physiologische wichtige Naturkonstanten noch nicht sicher ermittelt sind; es wäre wichtiger als manche andere Arbeit. *Die Theorie der Steinbildung, der Kalkablagerung in den Gefässen u.a. ist eine Phantasie, bevor nicht sicher ermittelt ist, ob der Kalk in Blut überhaupt übersättigt ist.*" (The italics are ours.)

Many attempts (2, 3, 4, 5) have been made to determine the solubility of $Ca_3(PO_4)_2$ by the simple method of dissolving this sa't in water, and analyzing the solution for its calcium and phosphorus content. Such a procedure is, however, quite inapplicable, since the salt hydrolyzes as rapidly as it dissolves, and the resulting solution is found to consist chiefly of the primary and secondary phosphates. A similar difficulty has been encountered in determining the solubility of the secondary phosphate $CaHPO_4$ by such direct methods. Hydrolysis occurs and the solution consists chiefly of primary phosphate. The hydrolytic reactions, to be sure, could be prevented by dissolving the tertiary phosphate in a strongly alkaline solution, in which case the phosphorus would exist almost entirely in the form of $PO_4^{\equiv}$ ion. The solubility, however, is so small that the resulting concentrations of calcium and total phosphorus in such a solution are far beyond the limits of analytical methods.[1]

Fortunately, for most purposes, the actual molar solubility is of little importance, for the solubility of dissociable substances is defined just as accurately and even more generally in terms of the solubility product. It has been shown by Nernst, and amply confirmed by others, that the product of the molar concentrations of the ions of any insoluble salt is a constant. Inasmuch as $Ca_3(PO_4)_2$ dissociates into $3Ca^{++}$ and $2PO_4^{\equiv}$ ions, the expression for the solubility product of this salt becomes:

$$[Ca^{++}]^3 \times [PO_4^{\equiv}]^2 = K_{s.p.} \tag{1}$$

[1] $[Ca^{++}]$ and $[PO_4^{\equiv}]$ are of the order of $10^{-6}$ molar.

By means of the solubility product principle one can evaluate and apply solubility data in systems where one or the other ion is present in excess. In the present case it enables us to work in acid solutions where the total calcium and total phosphate concentrations are large enough to be readily determined by analysis. In such solutions the $PO_4^{\equiv}$ ion is only a very small fraction of the total phosphate, but this minute fraction can be accurately determined[2] provided the pH of the solution and the ionization constants of $H_3PO_4$ are known. If such a system is in equilibrium with the solid phase $Ca_3(PO_4)_2$ we have met the condition necessary for evaluating the solubility product constant $K_{s.p.}$. A system suitable for this purpose was obtained by titrating 0.01 M $H_3PO_4$ with saturated lime water. Since the titration of phosphoric acid introduces certain points of unusual behavior a somewhat detailed description will be given.

II.

*Titration Curves of Phosphoric Acid.*

When a solution of $H_3PO_4$ is titrated with NaOH and the pH plotted against increments of added alkali, a characteristic curve is obtained which shows two sharp "breaks" as shown in Figs. 1 and 2. The first "break" occurs at a pH of 4.5 when 1 equivalent of $H_3PO_4$ has been neutralized by the NaOH. The solution here is essentially one of pure $NaH_2PO_4$ and is unbuffered; consequently small increments of alkali cause a great increase in alkalinity. Further additions of alkali permit the second hydrogen ion of the $H_3PO_4$ to dissociate in increasing quantities; a buffer mechanism is thus introduced, and the curve flattens out—only a small increase in pH results from the addition of alkali. A second sharp "break" in the curve at pH 8.5 occurs when the second hydrogen equivalent of $H_3PO_4$ has been completely neutralized. The solution now contains almost pure $Na_2HPO_4$; it is, therefore, again unbuffered and once more small increments of alkali cause a great increase in the pH of the solution. Beyond this point further additions of alkali cause relatively less increase

[2] See Tables II and III.

in pH, and the curve flattens out owing to two factors, namely the buffer action of the third hydrogen equivalent, which now becomes appreciable, and also because the pH of the solution approaches that of the alkali which is being added.

When $Ca(OH)_2$ is substituted for NaOH in such a titration, a curve of quite different character is obtained. The $Ca(OH)_2$ curve is identical with the NaOH curve up to and including the neutralization of the first equivalent of $H_3PO_4$. On the further addition of $Ca(OH)_2$ a precipitate appears in the solution and

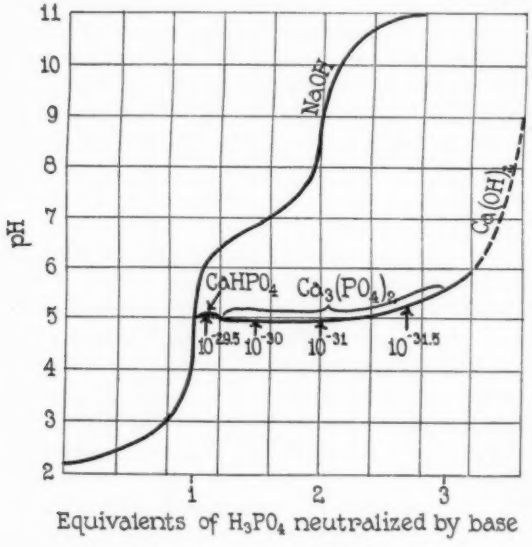


FIG. 1. Titration curves of $H_3PO_4$ at 38°C.

practically coincident with its first appearance the curve flattens out and continues almost horizontally at a pH just below 6 until nearly 3 equivalents of phosphoric acid have been neutralized. The curve then begins to rise, and shortly after the third equivalent has been neutralized it rises abruptly and again approaches the NaOH curve, as can be seen in Figs. 1, 2, and 3. The different course of the $Ca(OH)_2$ curve is obviously due to the precipitation of some form of calcium phosphate, the exact nature of which will be discussed below. When the concentration of calcium

rises to such a point as to permit the formation of a precipitate, further increments of $Ca(OH)_2$, instead of raising the pH, merely precipitate more phosphate. It is only when precipitation of the phosphate approaches completion that further additions of $Ca(OH)_2$ produce a rapid increase in pH.

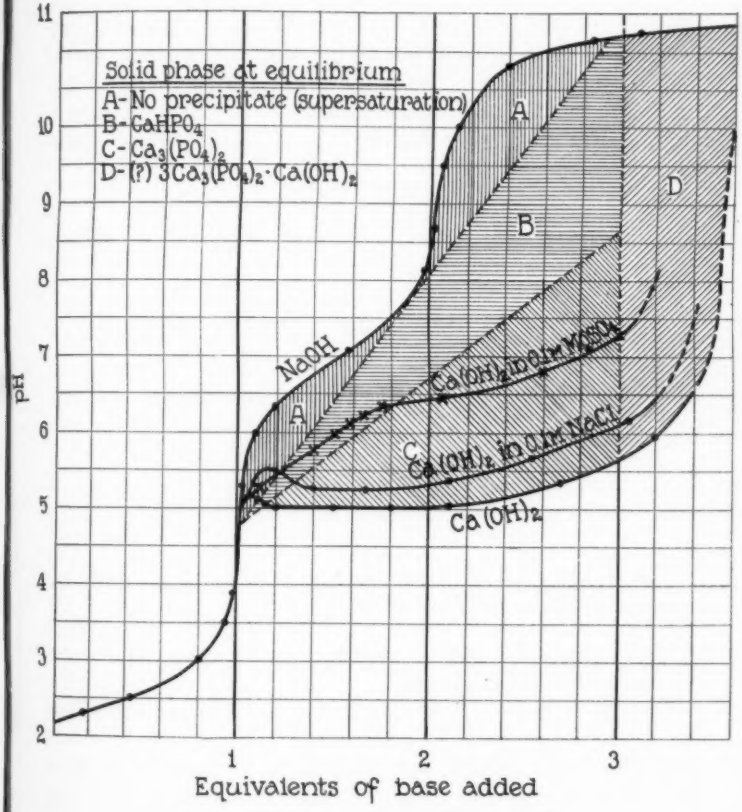


FIG. 2. Titration curves of $H_3PO_4$ at 38°C.

We have here a further buffer mechanism. In addition to the buffer effect produced by the acidic dissociations of $H_3PO_4$ as shown by the NaOH curve, we have the buffer mechanism due

to the formation of a precipitate. Thus, in those regions where a precipitate is formed, additions of $Ca(OH)_2$ exert an almost negligible effect upon the measured pH.

The area lying between the NaOH and $Ca(OH)_2$ curves is a measure of this additional buffer effect due to the formation of precipitate. The size of this area varies inversely with the solubility of the precipitate. The more highly insoluble the precipitate, the greater will be the area and the lower will be the $Ca(OH)_2$ titration curve, and, *vice versa*, any factor which increases the solubility of the precipitate will reduce the area and raise the titration curve, until, at the point where the precipitate

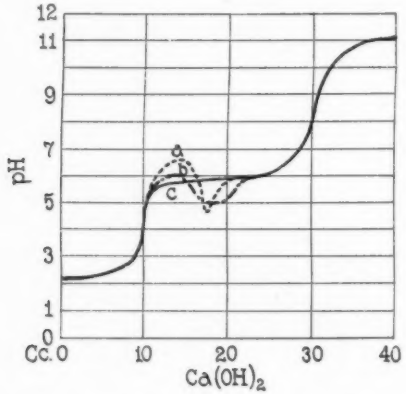


FIG. 3. Titration curves of $H_3PO_4$ with $Ca(OH)_2$, reproduced from Wendt and Clarke (6).

becomes completely soluble, the titration curve would become identical with the NaOH curve.

Wendt and Clarke (6) were the first to carry out such titrations, and we reproduce in Fig. 3 the curves which they described. They found that several days must elapse before equilibrium is reached in the region where a precipitate first appears, as shown by Curves *a*, *b*, and *c* representing readings made 3 minutes, 2 hours, and 10 days respectively after the addition of $Ca(OH)_2$. Curves *a* and *b* show a marked irregularity and exhibit the anomaly of increased acidity on the addition of lime water. Wendt and Clarke suggest as an explanation of these irregularities that the

first precipitate is $CaHPO_4$, which is unstable and is decomposed according to the equation:

(2) $$4CaHPO_4 \longrightarrow Ca_3(PO_4)_2 + Ca(H_2PO_4)_2$$

yielding the more insoluble tertiary phosphate and liberating the soluble primary acid phosphate. If their explanation is correct, it follows that the final precipitate at equilibrium should be $Ca_3(PO_4)_2$, and if so, such a system would prove an ideal one for evaluating the solubility product constant of $Ca_3(PO_4)_2$.

TABLE I.

*Titration of $H_3PO_4$ with $Ca(OH)_2$ at 38°C. (10 Days Continuous Shaking).*

| Equivalents of $Ca(OH)_2$ added. | Ca:P ratio* in precipitate. | pH | $[Ca^{++}]$ | [P] | $[H_2PO_4^-]$ | $[HPO_4^{=}]$ | $[PO_4^{\equiv}]$ | $K_{s.p.}[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$. | $-\log K_{s.p.}$ ($pK_{s.p.}$ for $Ca_3(PO_4)_2$). |
|---|---|---|---|---|---|---|---|---|---|
| | | | *m.ols* $\times 10^4$ | *m.ols* $\times 10^4$ | *m.ols* $\times 10^5$ | *m.ols* $\times 10^5$ | *m.ols* $\times 10^{12}$ | | |
| 1.09 | 1.26 | 5.08 | 57.0 | 100.0 | 99.0 | 11.9 | 5.71 | † | † |
| 1.20 | 1.96 | 5.04 | 50.2 | 90.1 | 89.0 | 9.73 | 4.28 | $2.31 \times 10^{-30}$ | 29.64 |
| 1.50 | 1.94 | 5.04 | 41.5 | 80.5 | 79.5 | 8.69 | 3.82 | $1.04 \times 10^{-30}$ | 29.98 |
| 1.80 | 1.91 | 5.00 | 34.4 | 65.1 | 64.4 | 6.44 | 2.58 | $2.71 \times 10^{-31}$ | 30.57 |
| 2.10 | 1.96 | 4.96 | 27.4 | 49.7 | 49.3 | 4.44 | 1.60 | $5.27 \times 10^{-32}$ | 31.28 |
| 2.70 | 1.93 | 5.31 | 13.7 | 22.0 | 21.6 | 4.40 | 3.59 | $3.32 \times 10^{-32}$ | 31.48 |
| 3.22 | 2.06 | 6.40(?) | 2.0 | 3.0 | | | | | |

* Theoretical Ca:P ratio for $CaHPO_4 = 1.29$; for $Ca_3(PO_4)_2 = 1.94$.

† No tertiary constant can be calculated, since the solid phase is $CaHPO_4$.

A preliminary electrometric titration at room temperature yielded a curve quite similar to Curve *c* of Wendt and Clarke. Their technique was followed as closely as possible. Since, however, we were primarily interested in determining the solubility product constant at body temperature (38°C.) more extensive experiments were undertaken at this temperature. When the identical procedure was followed at 38°C. the plateau of the titration curve was found to be somewhat lower than at room temperature, indicating that $Ca_3(PO_4)_2$, like other salts of calcium, is more insoluble at higher temperatures. However, an unexpected

difficulty was encountered at 38°C.; a striking irregularity in the curve, quite similar to that found in Curves *a* and *b* of Wendt and Clarke, persisted even after daily shaking for 2 months. As it seemed likely from the work of Wendt and Clarke that this was due to delayed equilibrium, an effort was made to hasten the attainment of equilibrium by continuous shaking. After 6 months intermittent shaking followed by 2 months continuous shaking only a small irregularity remained between the points represented by the addition of 1.05 to 1.20 equivalents of $Ca(OH)_2$. In this experiment it was necessary to use sterile technique to prevent the growth of moulds. A close approximation to these results was obtained after 10 days continuous shaking, and this procedure was therefore considered sufficiently accurate for our purpose.

A titration curve showing the results after 10 days continuous shaking is shown in Fig. 1. It is evident that beyond 1.20 equivalents of $Ca(OH)_2$ the curve is smooth.

In order to test the hypothesis of Wendt and Clarke, analyses of the solution and solid phase were made for various points on the curve. The results are given in Table I.

III.

*The Solid Phase at Equilibrium.*

From the analysis of the precipitates given in Table I and from other similar data which we have obtained, it is clear that the precipitate between 1.2 and about 3.0 equivalents of added $Ca(OH)_2$ is tertiary calcium phosphate (Ca:P ratio close to 1.94). Between 1.07 and 1.11 equivalents a crystalline precipitate[3] of $CaHPO_4$ (Ca:P ratio 1.29) is deposited.

[3] The only two stable varieties of secondary calcium phosphate which are well established are the anhydrous salt (monetite) and the dihydrate, $CaHPO_4 \cdot 2H_2O$ (brushite). The work of Bassett indicates that below 36°C. only the dihydrate, brushite, can coexist with the tertiary phosphate at equilibrium, while at higher temperatures only the anhydrous form, monetite, can coexist with $Ca_3(PO_4)_2$. The transition temperature was, however, determined by him with only approximate accuracy.

The crystals which we obtained have been examined through the courtesy of Mr. G. M. Hall of the Geology Department of the Johns Hopkins University to whom we wish to express our indebtedness. He finds that

Beyond 3 equivalents of $Ca(OH)_2$ a higher or more basic Ca:P ratio is obtained. The studies of Bassett (7) would indicate that this precipitate is hydroxyapatite for which he gives the formula $3Ca_3(PO_4)_2 \cdot Ca(OH)_2$. On the other hand, Cameron and Bell (4)

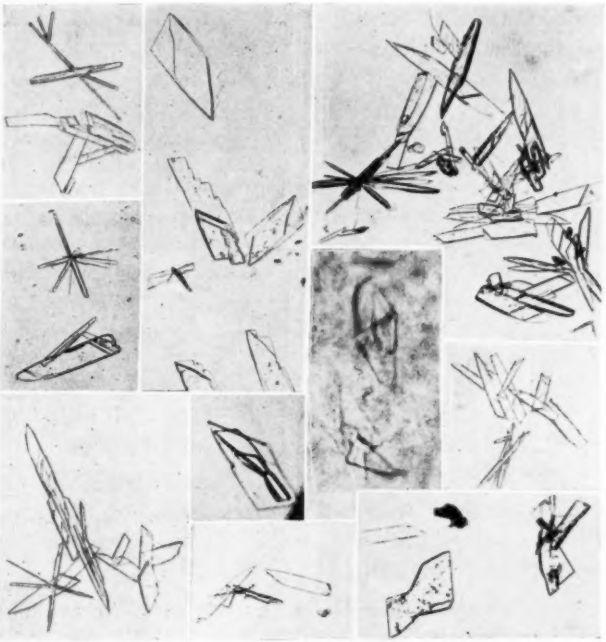

FIG. 4. Photomicrographs of crystals of $CaHPO_4 \cdot 2H_2O$.

believe that solid solutions of calcium oxide and tertiary phosphate exist, a conclusion which Bassett considers he has disproven.

Bassett's phase rule diagram (8) of the system $CaO\text{-}P_2O_5\text{-}H_2O$ also shows that for a given temperature there is only one com-

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they correspond with descriptions of brushite, $CaHPO_4 \cdot 2H_2O$, in specific gravity and in their index of refraction. The crystals belong to the monoclinic system while on the other hand the anhydrous salt yields crystals which belong to the triclinic system. Photomicrographs of the crystals which we obtained are given in Fig. 4.

position of the solution with which the solid phases secondary and tertiary calcium phosphate can coexist at equilibrium.

Our findings therefore confirm the hypothesis of Wendt and Clarke that the final precipitate throughout the greater part of the precipitation range is $Ca_3(PO_4)_2$ and we can thus employ the data obtained for the solutions in equilibrium with $Ca_3(PO_4)_2$ to calculate the solubility product constant for this salt.

IV.

*Calculation of the $PO_4^{\equiv}$ Ion Concentration.*

The formula used in calculating the $PO_4^{\equiv}$ ion concentration from the total phosphorus and hydrogen ion concentrations as determined analytically was derived as follows from the equations defining the first, second, and third ionization constants of $H_3PO_4$:

$$\frac{[H^+] \times [H_2PO_4^-]}{[H_3PO_4]} = K_1 \tag{3}$$

$$\frac{[H^+] \times [HPO_4^{=}]}{[H_2PO_4^-]} = K_2 \tag{4}$$

$$\frac{[H^+] \times [PO_4^{\equiv}]}{[HPO_4^{=}]} = K_3 \tag{5}$$

and the equation showing that the total phosphorus [P] is equal to the sum of the concentrations of the unionized and ionized forms of phosphoric acid:

$$[P] = [H_3PO_4] + [H_2PO_4^-] + [HPO_4^{=}] + [PO_4^{\equiv}]^{\ddagger} \tag{6}$$

Transposing equations (5), (4), and (3) we have:

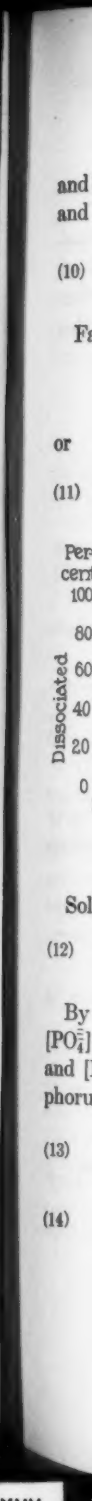
$$[HPO_4^{=}] = \frac{[H^+] \times [PO_4^{\equiv}]}{K_3} \tag{7}$$

$$[H_2PO_4^-] = \frac{[H^+] \times [HPO_4^{=}]}{K_2} = \frac{[H^+]^2 \times [PO_4^{\equiv}]}{K_2K_3} \tag{8}$$

$$[H_3PO_4] = \frac{[H^+] \times [H_2PO_4^-]}{K_1} = \frac{[H^+]^2 \times [HPO_4^{=}]}{K_1K_2} = \frac{[H^+]^3 \times [PO_4^{\equiv}]}{K_1K_2K_3} \tag{9}$$

Substituting in equation (6) the values for $[H_3PO_4]$, $[H_2PO_4^-]$,

‡ In these equations brackets signify molar concentrations.



and $[HPO_4^{=}]$ in terms of $[PO_4^{\equiv}]$ as obtained in equations (9), (8), and (7) we have:

$$(10)\quad [P] = \frac{[H^+]^3 \times [PO_4^{\equiv}]}{K_1K_2K_3} + \frac{[H^+]^2 \times [PO_4^{\equiv}]}{K_2K_3} + \frac{[H^+] \times [PO_4^{-}]}{K_3} + [PO_4^{\equiv}]$$

Factoring out $[PO_4^{\equiv}]$:

$$[P] = [PO_4^{\equiv}]\left[\frac{[H^+]^3}{K_1K_2K_3} + \frac{[H^+]^2}{K_2K_3} + \frac{[H^+]}{K_3} + 1\right]$$

or

$$(11)\quad [P] = [PO_4^{\equiv}]\left[\frac{[H^+]^3 + [H^+]^2 K_1 + [H^+] K_1K_2 + K_1K_2K_3}{K_1K_2K_3}\right]$$

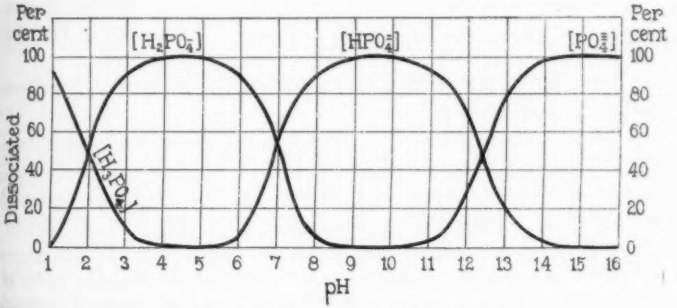



FIG. 5. Effect of pH on dissociation of $H_3PO_4$.

Solving for $[PO_4^{\equiv}]$:

$$(12)\quad [PO_4^{\equiv}] = \frac{[P]\, K_1K_2K_3}{[H^+]^3 + [H^+]^2 K_1 + [H^+] K_1K_2 + K_1K_2K_3}$$

By substituting in equations (7) and (8) the expression for $[PO_4^{\equiv}]$ from equation (12), expressions may be derived for $[HPO_4^{=}]$ and $[H_2PO_4^{-}]$ in terms of hydrogen ion concentration, total phosphorus, and the ionization constants of phosphoric acid:

$$(13)\quad [HPO_4^{=}] = \frac{[P]\,[H^+]\, K_1K_2}{[H^+]^3 + [H^+]^2 K_1 + [H^+] K_1K_2 + K_1K_2K_3}$$

$$(14)\quad [H_2PO_4^{-}] = \frac{[P]\,[H^+]^2 K_1}{[H^+]^3\,[H^+]^2 K_1 + [H^+] K_1K_2 + K_1K_2K_3}$$

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The relation between the hydrogen ion concentration and the ionization of phosphoric acid is shown graphically in Fig. 5 in which the percentage of the total phosphorus dissociated into each of the three types of phosphate ions is plotted against the pH. The values of $[H_2PO_4^-]$, $[HPO_4^=]$, and $[PO_4^{\equiv}]$ are obtained from equations (14), (13), and (12). For convenience in calculating, the fraction of the total phosphorus existing as $PO_4^{\equiv}$ ion is given as a function of pH in Tables II and III.

*Ionization Constants of $H_3PO_4$.*

Throughout this and the two succeeding papers the following values were taken for the ionization constants of $H_3PO_4$ at 38°C.:

$$K_1 = 1 \times 10^{-2}$$
$$K_2 = 1 \times 10^{-7}$$
$$K_3 = 4 \times 10^{-13}$$

We have been unable to find values[4] in the literature for these ionization constants at 38°C.

The values selected above are the means in round numbers of the best values obtained at 18° and 25°C. The values for $K_1$ vary from 0.7 (11) to 1.1 $\times$ $10^{-2}$ (10) for the dilutions employed in our work. Those for $K_2$ vary from 0.88 (12) to 1.95 (10) $\times$ $10^{-7}$. $K_3$ is less accurately known; the values given in the literature for this important constant are 3.6 (10) $\times$ $10^{-13}$ and 4 (13) $\times$ $10^{-13}$. *Since the same values of $K_1$, $K_2$, and $K_3$ are used throughout all of our calculations it is obvious that a revision of these figures by future workers will simply alter the values of $K_{s.p.}$ by a constant factor, but will not affect the conclusions which we have drawn.*

[4] Kugelmass and Shohl ((9), p. 662) state that:

"Abbott and Bray (10) found $K_7$, the second dissociation constant of phosphoric acid to be $2 \times 10^{-7}$ at 25° and $2.4 \times 10^{-7}$ at 38°."

We are not able to confirm this statement, for a study of the work of Abbott and Bray shows that their work was confined entirely to 18°C.

*Note Added in Proof.*—While this paper was in press, Dr. A. T. Shohl was kind enough to call our attention to the publication of Michaelis and Garmendia (Michaelis, L., and Garmendia, T., *Biochem. Z.*, 1914, lxvii, 431). These authors calculated a value of about $1 \times 10^{-7}$ at 38°C., using the van't Hoff equation, and obtained a value of about $2.4 \times 10^{-7}$ at 37° C. from hydrogen electrode measurements in the presence of 1 per cent NaCl Their curves show that the observed value at 37°C. should be close to $1 \times 10^{-7}$ in the absence of salt.

TABLE II.

*Relation between pH and the Fraction of Total Phosphate Ionized as $PO_4$.*

| pH | $[PO_4^{\equiv}]$ when [P] = 1. | p $[PO_4^{\equiv}]$ when [P] = 1. |
|---|---|---|
| 1 | $3.6 \times 10^{-19}$ | 18.44 |
| 2 | $2.0 \times 10^{-16}$ | 15.70 |
| 3 | $3.6 \times 10^{-14}$ | 13.44 |
| 4 | $4.0 \times 10^{-13}$ | 11.40 |
| 5 | $4.0 \times 10^{-10}$ | 9.40 |
| 6 | $3.6 \times 10^{-8}$ | 7.44 |
| 7 | $2.0 \times 10^{-6}$ | 5.70 |
| 8 | $3.6 \times 10^{-5}$ | 4.44 |
| 9 | $4.0 \times 10^{-4}$ | 3.40 |
| 10 | $4.0 \times 10^{-3}$ | 2.40 |
| 11 | $3.8 \times 10^{-2}$ | 1.42 |
| 12 | 0.28 | 0.55 |
| 13 | 0.80 | 0.10 |
| 14 | 0.98 | 0.01 |

TABLE III.

*Relation between pH and the Fraction of the Total Phosphate Ionized as $PO_4^{\equiv}$*

| pH | $[PO_4^{\equiv}]$ when [P] = 1. | p $[PO_4^{\equiv}]$ when [P] = 1. | pH | $[PO_4^{\equiv}]$ when [P] = 1. | p $[PO_4^{\equiv}]$ when [P] = 1. | pH | $[PO_4^{\equiv}]$ when [P] = 1. | p $[PO_4^{\equiv}]$ when [P] = 1. |
|---|---|---|---|---|---|---|---|---|
| 3.0 | $3.6 \times 10^{-14}$ | 13.44 | 5.0 | $4.0 \times 10^{-10}$ | 9.40 | 7.0 | $2.0 \times 10^{-6}$ | 5.70 |
| 3.1 | $5.9 \times 10^{-14}$ | 13.23 | 5.1 | $6.3 \times 10^{-10}$ | 9.20 | 7.1 | $2.8 \times 10^{-6}$ | 5.55 |
| 3.2 | $9.4 \times 10^{-14}$ | 13.02 | 5.2 | $9.9 \times 10^{-10}$ | 9.01 | 7.2 | $3.9 \times 10^{-6}$ | 5.41 |
| 3.3 | $1.5 \times 10^{-13}$ | 12.82 | 5.3 | $1.6 \times 10^{-9}$ | 8.81 | 7.3 | $5.3 \times 10^{-6}$ | 5.27 |
| 3.4 | $2.4 \times 10^{-13}$ | 12.61 | 5.4 | $2.5 \times 10^{-9}$ | 8.61 | 7.4 | $7.2 \times 10^{-6}$ | 5.14 |
| 3.5 | $3.9 \times 10^{-13}$ | 12.41 | 5.5 | $3.9 \times 10^{-9}$ | 8.41 | 7.5 | $9.6 \times 10^{-6}$ | 5.02 |
| 3.6 | $6.2 \times 10^{-13}$ | 12.21 | 5.6 | $6.1 \times 10^{-9}$ | 8.21 | 7.6 | $1.3 \times 10^{-5}$ | 4.90 |
| 3.7 | $1.0 \times 10^{-12}$ | 12.00 | 5.7 | $9.6 \times 10^{-9}$ | 8.02 | 7.7 | $1.7 \times 10^{-5}$ | 4.78 |
| 3.8 | $1.6 \times 10^{-12}$ | 11.80 | 5.8 | $1.5 \times 10^{-8}$ | 7.82 | 7.8 | $2.2 \times 10^{-5}$ | 4.66 |
| 3.9 | $2.5 \times 10^{-12}$ | 11.60 | 5.9 | $2.3 \times 10^{-8}$ | 7.63 | 7.9 | $2.8 \times 10^{-5}$ | 4.55 |
| 4.0 | $4.0 \times 10^{-12}$ | 11.40 | 6.0 | $3.6 \times 10^{-8}$ | 7.44 | 8.0 | $3.6 \times 10^{-5}$ | 4.44 |
| 4.1 | $6.3 \times 10^{-12}$ | 11.20 | 6.1 | $5.6 \times 10^{-8}$ | 7.25 | 8.1 | $4.7 \times 10^{-5}$ | 4.33 |
| 4.2 | $1.0 \times 10^{-11}$ | 11.00 | 6.2 | $8.7 \times 10^{-8}$ | 7.06 | 8.2 | $6.0 \times 10^{-5}$ | 4.22 |
| 4.3 | $1.6 \times 10^{-11}$ | 10.80 | 6.3 | $1.3 \times 10^{-7}$ | 6.88 | 8.3 | $7.6 \times 10^{-5}$ | 4.12 |
| 4.4 | $2.5 \times 10^{-11}$ | 10.60 | 6.4 | $2.0 \times 10^{-7}$ | 6.70 | 8.4 | $9.7 \times 10^{-5}$ | 4.01 |
| 4.5 | $4.0 \times 10^{-11}$ | 10.40 | 6.5 | $3.0 \times 10^{-7}$ | 6.52 | 8.5 | $1.2 \times 10^{-4}$ | 3.91 |
| 4.6 | $6.3 \times 10^{-11}$ | 10.20 | 6.6 | $4.5 \times 10^{-7}$ | 6.34 | 8.6 | $1.6 \times 10^{-4}$ | 3.81 |
| 4.7 | $1.0 \times 10^{-10}$ | 10.00 | 6.7 | $6.7 \times 10^{-7}$ | 6.17 | 8.7 | $2.0 \times 10^{-4}$ | 3.71 |
| 4.8 | $1.6 \times 10^{-10}$ | 9.80 | 6.8 | $9.8 \times 10^{-7}$ | 6.01 | 8.8 | $2.5 \times 10^{-4}$ | 3.60 |
| 4.9 | $2.5 \times 10^{-10}$ | 9.60 | 6.9 | $1.4 \times 10^{-6}$ | 5.85 | 8.9 | $3.1 \times 10^{-4}$ | 3.50 |
| 5.0 | $4.0 \times 10^{-10}$ | 9.40 | 7.0 | $2.0 \times 10^{-6}$ | 5.70 | 9.0 | $4.0 \times 10^{-4}$ | 3.40 |

The assumption that the ionization constants at 38°C. do not differ materially from those at 18° and 25° is justified by studies that have been made on many other weak acids at these temperatures.

## V.

### EXPERIMENTAL TECHNIQUE.

*Hydrogen Ion.*—Determinations were made electrometrically on all solutions[5] unless otherwise noted. A portable Leeds and Northrup potentiometer[6] was used with a Weston Standard cell. *Readings were made in triplicate and were discarded unless the potentials agreed within 1 millivolt* (0.02 pH). Determinations were carried out in a thermostat at 38°C.; and were standardized against 0.1 N HCl, the pH of which was taken as 1.040. A few readings were made at 37°; at this temperature the pH of 0.1 N HCl was taken to be 1.038. The determinations on blood serum were made by Cullen's method (14). The buffer solutions used were checked electrometrically.

*Calcium.*—When the amount of calcium was small the determinations were carried out using the micro method of Kramer and Tisdall (15). In solutions containing much calcium a modification of this method was employed, using proportionally larger quantities of solutions and reagents in 50 cc. centrifuge tubes. Determinations were checked frequently against a known calcium chloride solution.

*Phosphorus.*—When the quantity of phosphorus was small the analyses were made by the Briggs (16) modification of the Bell-Doisy method. With larger quantities a slight modification of

[5] Some difficulty was at first experienced in measuring the pH of solutions which had been in contact with a precipitate of calcium phosphate, for under these conditions the equilibrium between hydrogen and hydrogen ions is established very slowly. Electrode "poisoning" occurs quite rapidly at temperatures as high as 38°C.; thus it often happened that poisoning would become noticeable before hydrogen-hydrogen ion equilibrium had been attained. We were able to avoid this difficulty by soaking the electrode for half an hour at room temperature in a sample of the solution to be measured. When the electrode was then used at 38°C. equilibrium was established much more rapidly, and reproducible potentials could be obtained before any evidence of poisoning developed.

[6] Leeds and Northrup Co., Philadelphia, Catalog 75.

Fiske's method (17) was used. After the addition of magnesium citrate and ammonia, instead of shaking until crystallization is complete, as in Fiske's method, it was found more convenient to shake only until the first appearance of crystals. The solutions were then allowed to stand overnight. It was found that this method yielded larger crystals which could be more easily handled. By using 0.01 N NaOH for the final titration, an increased accuracy in reading the end-point was obtained.

*Handling of Precipitates.*—Solutions were filtered immediately upon removal from the thermostat through Whatman No. 40 filter paper. The precipitates were then washed twice with 95 per cent neutral alcohol and once with ether. After drying they were redissolved in dilute HCl for analysis.

*Reagents and Glassware.*—Pyrex flasks were used throughout the experiments, and in the subsequent handling of the precipitates and solutions. Pipettes and burettes were calibrated in the laboratory. The $H_3PO_4$ and $Ca(OH)_2$ used were Baker's C.P. chemicals.

## VI.

### *Solubility Product of $Ca_3(PO_4)_2$.*

From Table I it can readily be seen that the solubility product for tertiary calcium phosphate is far from being constant; on the contrary a progressive decrease in solubility occurs as more of the $H_3PO_4$ is neutralized by $Ca(OH)_2$. A study of the data given in the table, however, shows that aside from the $Ca^{++}$ and $PO_4^{\equiv}$ ions the only factors which change to any appreciable extent in these solutions are the concentrations of $H_2PO_4^-$ and $HPO_4^{=}$ ions. The concentration of $HPO_4^{=}$ is so small that the change in the absolute number of these ions is insignificant; on the other hand, the primary phosphate concentration is quite appreciable, and this ion is reduced from about 0.01 to about 0.002 M. We can thus consider that as more and more calcium hydroxide is added, the essential change in the solution is a decrease in the concentration of primary phosphate. Since these $K_{s.p.}$ values could be reproduced regularly, it was evident that their variation could not be due to experimental error but must be related to the change in the character of the solution due to this diminution of primary phosphate.

Many observers (18–23) have noted that the solubility and solubility products of difficultly soluble salts are increased by the addition of other salts to the solution. Inasmuch as it seemed likely that we were dealing here with a neutral salt effect, we undertook a study of the effect of a variety of neutral salts of different valence types upon the solubility product of $Ca_3(PO_4)_2$. It was found that the solubility of $Ca_3(PO_4)_2$ is enormously affected by neutral salts.

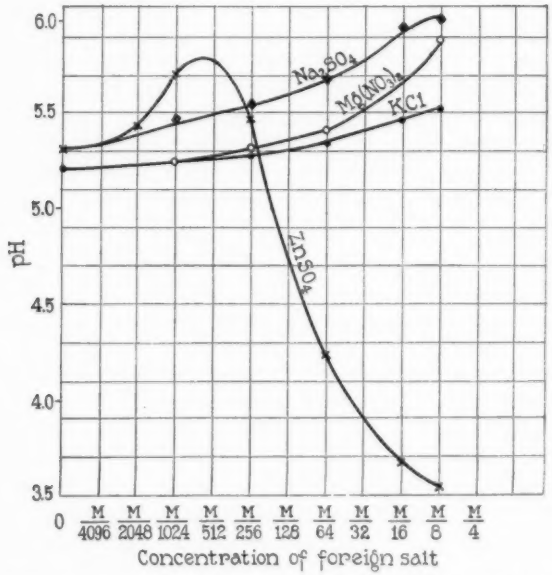


FIG. 6. Effect of foreign salts on the pH of saturated solutions of $Ca_3(PO_4)_2$.

This problem was attacked in two ways: experiments were carried out by selecting a definite point on the titration curve and introducing variable quantities of neutral salts, also by studying the titration curve of $H_3PO_4$ with $Ca(OH)_2$ in the presence of constant quantities of foreign salts.

In Fig. 2 are shown titration curves obtained in the presence of 0.1 M NaCl and 0.1 M $MgSO_4$. The curve in the presence of NaCl shows a plateau somewhat higher than the curve without salt addition. As explained previously this indicates an increased

solubility of the precipitate. The effect of $MgSO_4$ is considerably greater. The solid phase obtained at equilibrium in the various regions of the titration curves is shown by the shaded areas on the diagram. In Area *A* no visible precipitate was found during the 10 days of constant shaking. It seems likely that a state of supersaturation existed in this region. Area *B* represents the region in which the solid phase was found to be $CaHPO_4$, and Area *C* corresponds to a solid phase of $Ca_3(PO_4)_2$. The precipitates found in Area *D* yielded a Ca:P ratio always greater than 2.0 which indicates a more basic salt than $Ca_3(PO_4)_2$. This is in harmony with the work of Bassett (7), referred to previously, who obtained "hydroxyapatite," $3Ca_3(PO_4)_2 \cdot Ca(OH)_2$, from similar solutions. Since we were primarily interested in the secondary and tertiary phosphates, we did not extend our investigations into this area, but confined them to Areas *B* and *C*.[7] It is worthy of note that $CaHPO_4$ is the stable phase over a greater range of $Ca(OH)_2$ additions when foreign salts are present.

Fig. 6 shows the effect of adding salts of different valence types at a definite point on the titration curve. KCl and $Mg(NO_3)_2$ were added to a system in which 2.40 equivalents of $Ca(OH)_2$ had been added to $H_3PO_4$. The curves showing additions of $ZnSO_4$ and $Na_2SO_4$ do not coincide at zero salt addition, because the system to which these salts were added was prepared by adding 2.62 equivalents of $Ca(OH)_2$. The pH, and therefore the solubility of $Ca_3(PO_4)_2$, rises more rapidly the higher the valence type of the foreign salt added. The peculiar behavior of $ZnSO_4$ beyond M/1,024 concentration is due to a chemical rearrangement which occurs. A crystalline basic phosphate of zinc is precipitated and the $Ca_3(PO_4)_2$ is completely dissolved.

In Fig. 7 we have plotted the negative logarithm of the solubility product of $Ca_3(PO_4)_2$ which we shall designate as $pK_{s.p.}$ against the *dilution* of foreign salt added. This curve shows how striking is the effect of increasing the valence type of the foreign salt on the solubility.[8]

[7] The dotted lines bounding parts of these areas are hypothetical. The solid lines are experimental.

[8] Difficulty was encountered in determining the calcium in the presence of more than 0.001 M magnesium and zinc. The value given in Fig. 7 for 0.1 M $MgSO_4$ was obtained by determining the calcium in the precipitate, and calculating that in the solution by difference.

VII.

*Complete Dissociation and the Theory of Neutral Salt Effects.*[9]

In order to appreciate the cause of these striking salt effects, one must consider the theoretical basis upon which the solubility

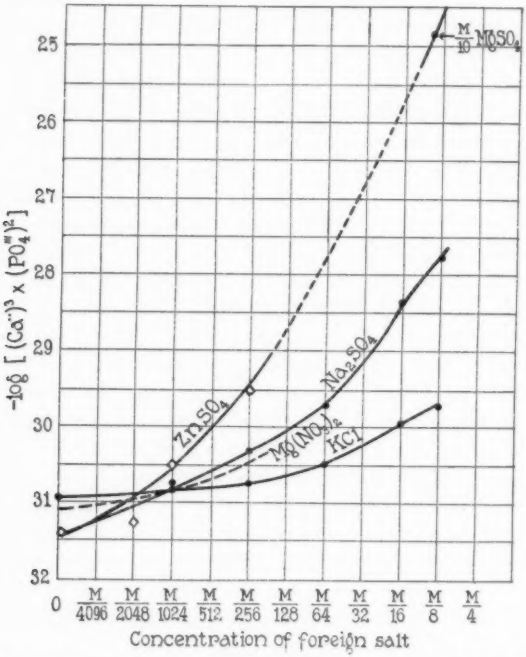


FIG. 7. Effect of foreign salts on the solubility of $Ca_3(PO_4)_2$ at 38°C.

product principle rests. This principle, as originally developed by Nernst from the law of mass action, and as commonly used, is only an approximation. Its validity is determined by the applicability of the gas laws to the solution. The law of mass action, and hence the solubility product principle, hold accurately

[9] For a more complete and more rigorous treatment of this subject, the reader is referred to Eucken, Fundamentals of physical chemistry, translated and adapted into English by Jette, E. R., and La Mer, V. K., New York, 1925, paragraphs 188*b* and *c* and 191*a*.

only under the ideal conditions of infinite dilution, where the space occupied by the particles of the solute is entirely negligible, and these particles may be considered as mathematical points. The particles are so far separated that they are free to move in any direction without interference from their neighbors. Under such ideal conditions the gas laws hold perfectly.

When, however, we consider finite concentrations of charged particles (ions) it is obvious that electrical complications are introduced. The particles are now much closer to each other and come within each other's sphere of attraction. Their motions are therefore restricted and they cannot exert their full osmotic pressure as calculated by the gas laws: $P = CRT$. We must therefore express the observed osmotic pressure by the relation:

$$P_{observed} = P_{ideal} - P_E \tag{15}$$

where $P_E$ is the electrical correction for this effect. This electrostatic effect restricting the motions of these charged particles not only diminishes the osmotic pressure which they exert, but it also affects their *active masses*[9] which determine the point of equilibrium of any chemical reaction. Since the mass law deals with *active* masses, it is evident that an electrical correction for this effect must be introduced if we are to express the mass law in the customary units of *concentration*. The expression for the mass law:

$$\frac{[C_A]\,[C_B]}{[C_{AB}]} = K \tag{16}$$

then becomes:

$$\frac{[f_A C_A]\,[f_B C_B]}{[f_{AB} C_{AB}]} = K \tag{17}$$

*where $f$ is the correction coefficient for all interfering effects including the electric effects of the charged particles.* The term $f$ has been designated the "activity coefficient," and represents the ratio of the *active* mass to the *total* mass or stoichiometric concentration. For convenience the term $f\,C$ is replaced by a single term $a$, which represents the true active mass, or the "activity." The corrected expression for the mass law then becomes:

$$\frac{[a_A]\,[a_B]}{[a_{AB}]} = K \tag{18}$$

When such a correction is introduced into the mass law, the law is valid, not only for the ideal conditions of infinite dilution, but for solutions of any concentration whatsoever.

Since the principle of the solubility product is a direct corollary of the mass law, it is obvious that the correct expression of the mass law (in terms of activities) should be used in deriving it. The expression for the solubility product then becomes:

$$[a_A]\,[a_B] = K_{s.p.} \quad (19)$$ §

§ Since the ordinary mass law expression for the ionization of a strong electrolyte involving the concentration of the undissociated molecule is incorrect, the unfortunate impression has been gained by many that the solubility product principle is not theoretically sound, but is essentially an empirical law (Stieglitz (13), p. 139 ff.) which holds in many cases because of two errors which in some way neutralize each other. Washburn (24) has, however, demonstrated that this view is quite erroneous and that the solubility product principle has a sound thermodynamic basis.

In a thermodynamic treatment the intermediate stages of a process do not need to be considered, provided that equilibrium exists between the initial and final stages. In a solubility equilibrium like that between solid $Ca_3(PO_4)_2$ and its ions, the presence of any undissociated molecules in solution (even supposing that such molecules do exist) may therefore be neglected, and the equilibrium equation may be written:

$$\text{Solid } Ca_3(PO_4)_2 \rightleftarrows 3Ca^{++} + 2PO_4^{\equiv\equiv\equiv} \quad (20)$$

We can therefore derive the solubility product principle as follows: At equilibrium the (Gibbs) chemical potentials or partial molal free energies ($\bar{F}$) of the substances composing this reversible process must be equal:

$$\bar{F}_{Ca_3(PO_4)_2} = 3\bar{F}_{Ca^{++}} + 2\bar{F}_{PO_4^{\equiv\equiv\equiv}} \quad (21)$$

or their algebraic sum must be equal to zero:

$$\bar{F}_{Ca_3(PO_4)_2} - 3\bar{F}_{Ca^{++}} - 2\bar{F}_{PO_4^{\equiv\equiv\equiv}} = 0$$

The relation between the chemical potential ($\bar{F}$) and the stoichiometric concentration ($C$) is expressed by the following equation when the gas laws hold:

$$\bar{F} = RT \ln C + i \quad (22)$$

where $i$ is a constant of integration.

When the gas laws do not hold we must replace the stoichiometric concentration ($C$) by the activity ($a$) and the relation becomes:

$$\bar{F} = RT \ln a + i \quad (23)$$

an equation by which the activity is best defined (20)

When expressed in terms of activities the solubility product remains a constant, not only under the ideal conditions of infinite dilution, but for concentrated solutions as well.

The question now arises: What value shall one take for the concentration of the ions to which the electrical correction is to be applied? Can one assume that the salt is completely dissociated, or must one first determine the degree of dissociation of the salt, and then apply the electrical correction to the fraction ionized?

In the past it has been generally assumed that strong electrolytes are not completely dissociated in solution. Thus for a salt like NaCl an equilibrium was supposed to exist between free ions and undissociated molecules as shown by the equation:

$$\text{(27)} \qquad NaCl \rightleftarrows Na^+ + Cl^- \tag{27}$$

This assumption is based on the fact that the osmotic pressure and other thermodynamic properties[10] of such a salt solution are not exactly doubled, as one would expect from the molar concentration if this salt were completely dissociated. The support for the classical view has always rested upon the fact that the degree of ionization, as calculated from the conductivity

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Substituting values in these terms for $\overline{F}$ in the above equation we get:

$$\text{(24)} \qquad RT \ln a_{Ca_3(PO_4)_2} - 3RT \ln a_{Ca^{++}} - 2RT \ln a_{PO_4^{\equiv}} + i_{Ca_3(PO_4)_2} - 3i_{Ca^{++}} - 2i_{PO_4^{\equiv}} = 0 \tag{24}$$

On rearranging and noting that the activity of the solid $Ca_3(PO_4)_2$ is constant:

$$\text{(25)} \qquad a^3_{Ca^{++}} \times a^2_{PO_4^{\equiv}} = e^{\left(\frac{i_{Ca_3(PO_4)_2} - 3i_{Ca^{++}} - 2i_{PO_4} + constant}{RT}\right)} \tag{25}$$

Since the solubility product is accurately defined by the exponential expression involving only constant factors, it is obviously a constant:

$$\text{(26)} \qquad a^3_{Ca^{++}} \times a^2_{PO_4^{\equiv}} = K_{s.p.} \tag{26}$$

The solubility product in stoichiometric units is likewise a thermodynamic necessity when the gas laws hold.

[10] Boiling point elevation, freezing point depression, electromotive force, etc.

ratio $\left(\frac{\Lambda_v}{\Lambda_\infty}\right)$, is in approximate agreement with the degree of ionization as calculated by thermodynamic methods. If this classical view is correct, it should follow that the law of mass action, when applied to an equilibrium like that given in equation (27) above, should yield a constant, irrespective of the method employed in determining the degree of dissociation. As a matter of fact it has long been known that the strong electrolytes, and particularly those of high valence type like $K_4Fe(CN)_6$ (25) are notorious in their disobedience to the classical mass law even in the highest dilutions. They constitute a distinct class from weak electrolytes, like acetic acid, which conform most beautifully to the Arrhenius theory (*i.e.* yield good ionization constants over a wide range of concentration). Although many attempts have been made to explain the anomalies of strong electrolytes by retaining the idea of incomplete dissociation, it must be admitted that most of these theoretical and experimental studies have only served to widen the gap which separates the strong and weak electrolytes.

Optical evidence convinced Bjerrum (26) in 1909 that an entirely new point of view must be taken; namely, that strong electrolytes are completely dissociated even in concentrated solutions. The trend of recent work is entirely in favor of this point of view. Bjerrum explains the reduction in the conductivity and thermodynamic properties of such solutions solely on the basis of the electrical forces between the ions which we have discussed above. Debye and Hückel (27) and Hückel (28) have recently succeeded in carrying out the difficult calculations of the magnitude of these effects, and their calculations are fully borne out by the experiments of Brönsted and La Mer (19). This result is a gratifying one as it is also in complete agreement with the X-ray studies of Bragg and Bragg (29) and of Debye and Scherrer (30), who have shown that undissociated molecules of typical salts do not exist *even in the solid state.* They find that a crystal of a salt like NaCl is composed wholly of ions of $Na^+$ and $Cl^-$ arranged in a space lattice. The function of the solvent is simply to furnish a medium in which the ions already existent in the solid state may be dispersed, and therefore the ions in solution can carry a current since they are now free to move.

Inasmuch as the various lines of evidence now point to the complete dissociation of strong electrolytes, it is clear that we should apply the electrical correction to the stoichiometric ion concentration, rather than to some fraction which might be assumed to be ionized.[11]

From what has been said about electrostatic effects between ions in solutions of finite concentrations, it is easy to understand why the introduction of foreign salts should have such profound effects on solubility. The introduction of charged particles (ions of foreign salt) sets up additional electrostatic forces, and the activities of the ions of the insoluble salt are thereby diminished. The collisions[12] made by the ions of this salt are either reduced in number or made less effective, and the tendency for precipitation to occur (which is dependent on the number of effective collisions) is therefore correspondingly reduced. A greater stoichiometric concentration of reacting ions is thus required to provide the number of effective collisions necessary to maintain the solubility equilibrium, and, although the solubility product in terms of activities is uninfluenced by salt additions, the solubility product in terms of the older units—stoichiometric concentrations—is considerably increased.[13]

VIII.

*Effect of Valency and the Concept of Ionic Strength.*

The effect of valency remains to be explained. It is well known that such salt effects increase rapidly in magnitude as the *valence type of the insoluble salt* is increased. The experiments which we have reported bring additional evidence to show that the salt

[11] In a purely thermodynamic treatment, such as that developed by Brönsted (20-23), it is immaterial whether one assumes complete dissociation or not, since even with weak electrolytes which are incompletely dissociated we may still employ the activity concept. In this case the stoichiometric activity coefficient measures both the true degree of dissociation and the electric effects. Brönsted, however, pointed out that *all of his results on strong electrolytes* are most simply interpreted on the basis of complete dissociation.

[12] Between the ions in solution and the solid phase composed of ions in a space lattice.

[13] *I.e.*, $K_{s.p.}$ increases in absolute value and its negative logarithm, $pK_{s.p.}$ decreases.

effect is also greatly increased as the *valence type of the foreign salt* increases. Since, however, the valence of an ion is nothing more or less than the number of excess electrical charges on that ion, we can readily see why the electrostatic force, and hence the electrical correction, will increase markedly with the valence of the ions either of the foreign salt or of the insoluble salt.

Coulomb's law for the electrical force between charged particles states that:

$$\text{Force} = \frac{e_1 e_2}{Dr^2} \tag{28}$$

Where $D$ is the dielectric constant of the medium, $r$ is the distance between the charged particles, and $e_1$ and $e_2$ are the electrical charges of the particles themselves. When applied to ions it is evident that the electrostatic force depends upon the product of the valences of the ions concerned; it is thus evident why the effect increases so rapidly with the valence of the ions of either the foreign salt or the insoluble salt. When the valence of the ions involved is identical, the formula becomes:

$$\text{Force} = \frac{e^2}{Dr^2} \tag{29}$$

Although it does not follow directly that the electrical corrections should vary with the square of the valence, still it can be shown[14] (31) at least for *dilute* solutions, that this is the case; the solubility effects of foreign salt additions depend on the square of the valences of the ions of the insoluble salt, and the square of the valences of the ions of soluble salts in the solution.

The conception of the "ionic strength" of a solution developed by Lewis and Randall (32) is in harmony with this point of view. According to these authors the ionic strength ($\mu$) is defined as one-half the sum of the molar concentrations of each ion, multiplied by the square of its valence. If the molarity of the ion is designated by $m$ and its valence by $z$, the relation may be expressed by the equation:

$$\mu = \tfrac{1}{2}(m_1 z_1^2 + m_2 z_2^2 + m_3 z_3^2 \ldots\ldots\ldots\ldots) \tag{30}$$
$$= \tfrac{1}{2}\Sigma m z^2$$

[14] Brönsted and La Mer (19), p. 556.

The advantage of using the ionic strength is that it takes account of the effect of the different valences of ions in solvent salt mixtures, and, according to Lewis and Randall, we have the general principle that "*in dilute solutions the activity coefficient of a strong electrolyte is the same in all solutions of the same ionic strength.*" In other words, solutions of the same ionic strength, no matter what their components are, should produce identical changes in the solubility of any particular insoluble salt.

Although the conception of ionic strength was entirely empirical as far as Lewis and Randall were concerned, Debye and Hückel (28) have shown independently that the ionic strength has a sound theoretical basis as well as being in agreement with known facts. Brönsted and La Mer (19) have shown that the comp'icated expressions of Debye and Hückel may be reduced to the comparatively simple formula:

$$-\log_{10} f_{(salt)} = \alpha' z_1 z_2 \sqrt{\mu} \tag{31}$$

where $z_1$ and $z_2$ are the valences of the cation and anion of the difficultly soluble (saturating) salt, *$\alpha'$ is a universal constant*[15] having a value of 0.50, and $\mu$ has the significance defined above. This simple formula takes account of variations in the activity coefficient due to the electrical effects which we have previously discussed, and is valid when the ionic strength does not exceed $0.01\mu$.

When we come to more concentrated solutions, however, this simple formula is no longer adequate, since *secondary electrical effects* now come into play. In such concentrated solutions, ions can no longer be considered as charged points, but must be considered as spheres of finite size[16] over whose surface the electrical charge is distributed. This factor and an additional secondary electrical effect, termed the "salting-out" or "hydration" effect, are taken care of by introducing into the above formula a correction term, $\beta\mu$, so that we have:

$$-\log_{10} f_{(salt)} = \alpha' z_1 z_2 \sqrt{\mu} + \beta\mu \tag{32}$$

an equation which is valid up to ionic strengths as great as 0.1 $\mu$

[15] The $\alpha'$ of this paper is $\frac{3\alpha}{2.303}$ of Brönsted and La Mer (19).

[16] The calculations of Debye and Hückel show that the ionic size necessary to account for this secondary electric effect turns out to be approximately that of the actual diameter of the ions; namely, $1 - 5 \times 10^{-8}$ cm.

or more. Although the introduction of such a linear correction is theoretically sound, the value of the constant, $\beta$, must, for the present at least, be determined empirically, since we have no reliable independent means of measuring the size or "hydration" of ions.

IX.

*Solubility Product and Activity Coefficient of $Ca_3(PO_4)_2$ in Salt Solutions.*

It has been pointed out previously that the solubility product of any insoluble salt is a constant under all conditions *when this product is expressed in terms of activities.* For tertiary calcium phosphate this relation may be written:

$$[a_{Ca^{++}}]^3 \times [a_{PO_4^{\equiv}}]^2 = a_{K_{s.p.}} \tag{26}$$

If it were possible to measure these ion activities directly (as, for instance, by electrometric methods similar to those in use for hydrogen ions, chlorine ions, etc.) we should not be so greatly concerned about salt effects or activity coefficients, for we should then have a standard for determining supersaturation or undersaturation with $Ca_3(PO_4)_2$ in any medium whatsoever. Unfortunately, however, such methods have not yet been perfected and we are forced to employ the activity coefficient of the salt (obtained from the change in the stoichiometric solubility product) in various salt solutions, before we can accurately define the solubility in any particular medium.

A very considerable simplification is, however, introduced now that we have a theory relating the changes in solubility (and hence, activity coefficients) with the salt concentrations of the medium. The theory of Debye and Hückel accounts mathematically (within certain limits) for the effects of salts on solubility, as far as it has hitherto been tested. Since, however, the theory has never been tested with insoluble salts of such a high valence type as tertiary calcium phosphate, or for cases where a weak electrolyte like $PO_4^{\equiv}$ ion is involved, it seemed worth while to see if the variations in the solubility of $Ca_3(PO_4)_2$ which we observed in different salt solutions would conform to the predictions of the theory. If this should prove to be the case, we would have not only added evidence for the general validity of the Debye-Hückel theory, but we would

be able to predict, within certain limits, the stoichiometric solubility (or the activity coefficient) of $Ca_3(PO_4)_2$ in a medium of any known ionic strength.

The data from our experiments on the solubility of $Ca_3(PO_4)_2$¶ in salt solutions are given in Tables IV to X inclusive. The column designated $\Delta$ $pK_{s.p.}$ represents the difference between $pK_{s.p.}$ observed and $pK_{s.p.}$ at infinite dilution. The ionic strength ($\mu$) is calculated from the formula of Lewis and Randall given above, and the activity coefficient of the salt ($f$) is calculated from the relation:

$$-\log_{10} f_{(salt)} = \frac{\Delta pK_{s.p.}}{\nu} \quad (33)\text{‖}$$

where $\nu$ represents the number of ions of the insoluble salt. Table IV represents excesses of primary calcium phosphate; Tables V, VI, VII, and VIII show the effects of additions of KCl, NaCl,

¶ Our treatment is not entirely consistent in one respect. In determining the values of the phosphate ions from the stoichiometric equations (12), (13), and (14) we have used the activity of the hydrogen ion $a_{H^+}$ as measured by the hydrogen electrode, instead of the stoichiometric $[H^+]$ concentration.

We have therefore neglected the comparatively slight changes in the activity coefficient of the hydrogen ion as we pass from one salt solution to another. In view of the fact that $H^+$ is univalent the error so introduced is very small.

‖ The relation: $-\log_{10} f_{(salt)} = \frac{\Delta\, pK_{s.p.}}{\nu}$ may be derived as follows:

The activity coefficient of the salt is defined by the equation:

$$f_{(salt)} = \frac{S}{S_\infty}$$

where $S$ is the observed molar solubility and $S_\infty$ is the solubility at infinite dilution. The relation between the molar solubility and the solubility product (*cf.* Brönsted and La Mer(19), p. 564) is given by the equation:

$$S = \sqrt[\nu]{K_{s.p.}}$$

where $\nu$ is the number of ions composing the salt. From this it follows that the activity coefficient may be expressed in terms of solubility products as follows:

$$f_{(salt)} = \frac{\sqrt[\nu]{K_{s.p.}\text{ observed}}}{\sqrt[\nu]{K_{s.p.}\text{ at infinite dilution}}} = \sqrt[\nu]{\frac{K_{s.p.}\text{ observed}}{K_{s.p.\ \infty}}}$$

$Na_2SO_4$, $ZnSO_4$, and $MgSO_4$ to systems saturated with $Ca_3(PO_4)_2$. Table IX gives data obtained from titrating $H_3PO_4$ with $Ca(OH)_2$ in a medium containing inorganic salts in approximately the concentrations found in blood serum.

In Fig. 8 are shown solubility curves in various salt solutions plotted against the square root of the ionic strength of the medium ($\sqrt{\mu}$). The straight line running diagonally across the figure represents the change in solubility product with ionic strength, calculated from the Debye-Hückel equation as simplified by Brönsted and La Mer:

$$\frac{\Delta pK_{s.p.}}{\nu} = -\log_{10} f_{(salt)} = \alpha' z_1 z_2 \sqrt{\mu} \tag{34}$$

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Taking the negative logarithm of both sides of this equation:

$$-\log_{10} f_{(salt)} = -\frac{1}{\nu}\log_{10}\frac{K_{s.p.}\text{ observed}}{K_{s.p.\ \infty}} = \frac{1}{\nu}[-\log_{10} K_{s.p.}\text{ observed} - (-\log_{10} K_{s.p.\ \infty})]$$

Substituting for $-\log_{10} K_{s.p.}$ its equivalent $pK_{s.p.}$ we get:

$$-\log_{10} f_{(salt)} = \frac{pK_{s.p.}\text{ observed} - pK_{s.p.\ \infty}}{\nu}$$

If we define $\Delta pK_{s.p.}$ as the difference between the observed $K_{s.p.}$ and the $K_{s.p.}$ at infinite dilution, we may then write:

$$-\log_{10} f_{(salt)} = \frac{\Delta\ pK_{s.p.}}{\nu}$$

When we speak of the activity coefficient of the salt we do not necessarily imply the existence of undissociated salt molecules in solution. By the activity coefficient of the salt is meant simply the geometric mean of the activity coefficient of the ions which compose it. In the case of $Ca_3(PO_4)_2$ the relation between the activity coefficients of the ions and the salt is defined by the equation:

$$f^3_{Ca^{++}} \times f^2_{PO_4^{\equiv}} = f^5_{Ca_3(PO_4)_2}$$

TABLE IV.

*Effect of Varying Concentrations of Primary Calcium Phosphate* on Solubility of $Ca_3(PO_4)_2$ at 38°C.*

TABLE IV.

*Effect of Varying Concentrations of Primary Calcium Phosphate* on Solubility of $Ca_3(PO_4)_2$ at 38°C.*

| Serial No. | Equivalents of $Ca(OH)_2$ added. | pH | $[Ca^{++}]$ | [P] | $[H_2PO_4^-]$ | $[HPO_4^=]$ | $[PO_4^{\equiv}]$ | $K_{s.p.} \times 10^{28}$. | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ $(32.5-pK_{s.p.})$. | $\mu$ | $\sqrt{\mu}$ | $\sqrt{\mu}+\beta\mu$† | $\sqrt[5]{Ca_3(PO_4)_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^5$ | *mols* $\times 10^{12}$ | | | | | | | |
| M 5 | 1.23 | 5.03 | 5.00 | 10.3 | 10.2 | 10.9 | 4.66 | 272.5 | 29.57 | 2.9 | 0.0153 | 0.123 | 0.181 | 0.26 |
| M 6 | 1.36 | 4.94 | 4.55 | 10.3 | 10.2 | 8.9 | 3.10 | 90.5 | 30.04 | 2.5 | 0.0144 | 0.120 | 0.175 | 0.32 |
| M 7 | 1.50 | 4.86 | 4.15 | 8.39 | 8.32 | 6.0 | 1.74 | 21.8 | 30.66 | 1.8 | 0.0126 | 0.112 | 0.160 | 0.44 |
| M 8 | 1.63 | 4.84 | 4.00 | 7.74 | 7.67 | 5.3 | 1.47 | 13.8 | 30.86 | 1.6 | 0.0119 | 0.109 | 0.154 | 0.48 |
| M 9 | 1.77 | 4.87 | 3.50 | 6.97 | 6.91 | 5.1 | 1.52 | 9.91 | 31.00 | 1.5 | 0.0105 | 0.103 | 0.143 | 0.50 |
| CC 1 | 1.20 | 5.04 | 5.02 | 9.01 | 8.90 | 9.73 | 4.28 | 231 | 29.64 | 2.9 | 0.0148 | 0.122 | 0.178 | 0.26 |
| CC 2 | 1.50 | 5.04 | 4.15 | 8.05 | 7.95 | 8.69 | 3.82 | 104 | 29.98 | 2.5 | 0.0126 | 0.112 | 0.160 | 0.32 |
| CC 3 | 1.80 | 5.00 | 3.44 | 6.51 | 6.44 | 6.44 | 2.58 | 27.1 | 30.57 | 1.9 | 0.0102 | 0.101 | 0.140 | 0.42 |
| CC 4 | 2.10 | 4.955 | 2.74 | 4.97 | 4.93 | 4.44 | 1.60 | 5.27 | 31.28 | 1.2 | 0.0070 | 0.0836 | 0.110 | 0.58 |
| CC 5 | 2.40 | 5.18 | 2.03 | 2.90 | 2.86 | 4.32 | 2.62 | 5.74 | 31.24 | 1.3 | 0.0056 | 0.0753 | 0.0966 | 0.55 |
| CC 6 | 2.70 | 5.31 | 1.37 | 2.20 | 2.16 | 4.40 | 3.59 | 3.32 | 31.48 | 1.0 | 0.0039 | 0.0625 | 0.0773 | 0.63 |
| DD 1 | 2.39 | 5.15 | 1.65 | 4.32 | 4.26 | 6.01 | 3.39 | 5.16 | 31.29 | 1.2 | 0.0056 | 0.0746 | 0.0959 | 0.58 |

*Preparation of Solutions.*

*Series M.*—To 21.6 cc. of 0.1 M $H_3PO_4$ was added 0.02 M $Ca(OH)_2$ until the appropriate number of equivalents had been added. Solutions were then made up to 200 cc. with distilled water. Intermittent shaking for 6 months, followed by 2 months continuous shaking. Temperature 38°C. Sterile technique followed throughout.

*Series CC.*—To 19.6 cc. of 0.1 M $H_3PO_4$ was added 0.02 M $Ca(OH)_2$ until the appropriate number of equivalents had been added. The solution was then made up to 200 cc. with distilled water. Shaken continuously for 10 days at 38°C.

*Series DD.*—See data on experiments with additions of $ZnSO_4$.

* Slight variations in the concentrations of other ions occurred in these experiments, but only the $Ca^{++}$ and $H_2PO_4^-$ ions varied enough to affect appreciably the ionic strength of the solution ($\mu$).

† Value of $\beta$ taken as 3.8.

TABLE V.

*Effect of Additions of KCl and NaCl on Solubility of $Ca_3(PO_4)_2$ at 38°C.*

| Serial No. | pH | $[Ca^{++}]$ | $[Na^+]$ | $[K^+]$ | $[Cl^-]$ | [P] | $[H_2PO_4^-]$ | $[HPO_4^{--}]$ | $[PO_4^{---}]$ | $K_{s.p.} \times 10^{25}$. | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ $(32.5-pK_{s.p.})$. | $\mu$ | $\sqrt{\mu}$ | $\sqrt{\mu} + \beta\mu$* | $f_{Ca_3(PO_4)_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *m.ols* $\times 10^3$ | *m.ols* $\times 10^3$ | *m.ols* $\times 10^3$ | *m.ols* $\times 10^3$ | *m.ols* $\times 10^3$ | *m.ols* $\times 10^3$ | *m.ols* $\times 10^5$ | *m.ols* $\times 10^{13}$ | | | | | | | |
| V 1 | 5.21 | 1.95 | | | | 3.70 | 3.64 | 5.90 | 3.83 | 10.9 | 30.96 | 1.5 | 0.0058 | 0.076 | | 0.50 |
| V 2 | 5.27 | 1.975 | | 3.9 | 3.91 | 3.62 | 3.55 | 6.61 | 4.92 | 18.7 | 30.73 | 1.8 | 0.0098 | 0.099 | 0.086 | 0.44 |
| V 3 | 5.33 | 1.99 | | 15.6 | 15.6 | 3.56 | 3.49 | 7.44 | 6.37 | 32.0 | 30.50 | 2.0 | 0.0265 | 0.163 | 0.128 | 0.40 |
| V 4 | 5.46 | 2.06 | | 62.5 | 62.5 | 3.52 | 3.43 | 9.87 | 11.4 | 113 | 29.94 | 2.6 | 0.0744 | 0.273 | 0.176 | 0.30 |
| V 5 | 5.51 | 2.10 | | 125 | 125 | 3.516 | 3.40 | 11.0 | 14.3 | 188 | 29.73 | 2.8 | 0.131 | 0.363 | 0.203 | 0.28 |
| V 6 | 5.52 | 2.20 | 125 | | 125 | 3.56 | 3.45 | 11.5 | 15.1 | 244 | 29.61 | 2.9 | 0.131 | 0.363 | 0.219 | 0.26 |
| T 7 | 5.53 | 3.07 | 250 | | 250 | 3.99 | 3.86 | 13.1 | 17.7 | 906 | 29.04 | 3.5 | 0.259 | 0.509 | 0.224 | 0.20 |

*Preparation of Solutions.*

2.40 equivalents and $Ca(OH)_2$ added. Solutions prepared by mixing 20.7 cc. of 0.1 M $H_3PO_4$ and 123.97 cc. of 0.02 M $Ca(OH)_2$. The appropriate amount of NaCl or KCl was added and the volume made up to 200 cc. with distilled water. Shaken 8 days at 38°C.

* Value of $\beta$ taken as $-1.3$ for KCl and $-1.1$ for NaCl.

TABLE VI.

*Effect of Additions of $Na_2SO_4$ on Solubility of $Ca_3(PO_4)_2$ at 38°C.*

| Serial No. | pH | $[Ca^{++}]$ | $[Na^+]$ | $[SO_4^{--}]$ | [P] | $[H_2PO_4^-]$ | $[HPO_4^{--}]$ | $[PO_4^{---}]$ | $K_{s.p.} \times 10^{29}$ | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ $(32.5\sqrt{\mu}-pK_{s.p.})$ | $\mu$ | $\sqrt{\mu}$ | $\sqrt{\mu}+\beta\mu^*$ | $f_{Ca_3(PO_4)_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^6$ | *mols* $\times 10^3$ | *mols* $\times 10^5$ | *mols* $\times 10^{12}$ | | | | | | | |
| Z 1 | 5.30 | 1.46 | | | 2.19 | 2.15 | 4.29 | 3.42 | 3.61 | 31.44 | 1.1 | 0.00408 | 0.0640 | | 0.60 |
| Z 2 | 5.46 | 1.475 | 1.95 | 0.97 | 2.37 | 2.31 | 6.65 | 7.68 | 18.9 | 30.72 | 1.8 | 0.00712 | 0.0845 | 0.079 | 0.44 |
| Z 3 | 5.55 | 1.56 | 7.81 | 3.95 | 2.42 | 2.34 | 8.30 | 11.8 | 52.8 | 30.28 | 2.2 | 0.0162 | 0.127 | 0.114 | 0.36 |
| Z 4 | 5.67 | 1.66 | 31.25 | 15.5 | 2.51 | 2.40 | 11.2 | 21.0 | 201 | 29.70 | 2.8 | 0.0516 | 0.227 | 0.186 | 0.28 |
| Z 5 | 5.94 | 2.00 | 125 | 62.5 | 2.755 | 2.54 | 22.1 | 76.9 | 4,730 | 28.33 | 4.2 | 0.197 | 0.445 | 0.289 | 0.145 |
| Z 6 | 5.99 | 2.44 | 250 | 125 | 3.055 | 2.77 | 27.2 | 106.3 | 16,400 | 27.79 | 4.7 | 0.382 | 0.619 | 0.317 | 0.115 |

*Preparation of Solutions.*

2.62 equivalents of $Ca(OH)_2$ added. Solutions were prepared from 128.5 cc. of 0.02 M $Ca(OH)_2$ and 19.6 cc. of 0.1 M $H_3PO_4$. The appropriate amounts of $Na_2SO_4$ solution were then added and the volume was make up to 200 cc. with distilled water. Continuous shaking for 8 days at 38°C.

* Value of $\beta$ taken as $-0.79$.

TABLE VII.

*Effect of Additions of $ZnSO_4$ on Solubility of $Ca_3(PO_4)_2$ at 38°C.*

| Serial No. | Equivalents of $Ca(OH)_2$ added. | pH | $[Ca^{++}]$ | $[Zn^{++}]$ | [p] | $[H_2PO_4^-]$ | $[HPO_4^{--}]$ | $[PO_4^{---}]$ | $[SO_4^{--}]$ | $K_{s.p.} \times 10^{32}$. | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ $(32.5\text{-}pK_{s.p.})$. | $\mu$ | $\sqrt{\mu}$ | $\sqrt{\mu} + \beta\mu$* | $f_{Ca_3(PO_4)_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mols* $\times 10^3$ | *mols* $\times 10^4$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^{11}$ | *mols* $\times 10^4$ | | | | | | | |
| Z 1 | 2.62 | 5.30 | 1.46 | | 2.19 | 2.15 | 4.29 | 3.42 | | 3.61 | 31.44 | 1.1 | 0.0041 | 0.064 | | 0.60 |
| Z 7 | 2.62 | 5.42 | 1.19 | 4.88 | 2.06 | 2.00 | 5.27 | 5.55 | 4.88 | 5.21 | 31.28 | 1.2 | 0.0055 | 0.074 | 0.126 | 0.58 |
| Z 8 | 2.62 | 5.695 | 1.01 | 9.77 | 1.94 | 1.85 | 9.15 | 18.1 | 9.77 | 33.8 | 30.47 | 2.0 | 0.0070 | 0.084 | 0.150 | 0.40 |
| DD 1 | 2.39 | 5.15 | 1.65 | | 4.32 | 4.26 | 6.01 | 3.39 | | 5.16 | 31.29 | 1.2 | 0.0056 | 0.075 | | 0.58 |
| DD 2 | 2.39 | 5.44 | 1.98 | 4.88 | 4.29 | 4.17 | 11.5 | 12.66 | 4.88 | 123 | 29.91 | 2.6 | 0.0082 | 0.091 | 0.168 | 0.30 |
| DD 3 | 2.39 | 5.51 | 2.20 | 9.77 | 3.87 | 3.75 | 12.1 | 15.7 | 9.77 | 262 | 29.58 | 2.9 | 0.0104 | 0.103 | 0.201 | 0.26 |

*Preparation of Solutions.*

*Series Z.*—128.5 cc. of 0.02 M $Ca(OH)_2$ were mixed with 19.6 cc. of 0.1 M $H_3PO_4$. After adding the appropriate amount of $ZnSO_4$ the volume was made up to 200 cc. with distilled water. Continuously shaken for 9 days at 38°C.

*Series DD.*—121.9 cc. of 0.02 M $Ca(OH)_2$ were mixed with 20.4 cc. of 0.1 M $H_3PO_4$. After adding the appropriate amount of $ZnSO_4$ the volume was made up to 200 cc. with distilled water. Continuous shaking was carried out for 14 days at 38°C.

* Value of $\beta$ taken as 9.4.

TABLE VIII.

*Effect of Addition of $MgSO_4$ on Solubility of $Ca_3(PO_4)_2$ at 38°C.*

| Serial No. | pH | $[Ca^{++}]$ | $[Mg^{++}]$ | $[SO_4^{--}]$ | $[P]$ | $[H_2PO_4^-]$ | $[HPO_4^{--}]$ | $[PO_4^{---}]$ | $K_{s.p.} \times 10^{25}$ | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ $(32.5-pK_{s.p.})$. | $\mu$ | $\sqrt{\mu}$ | $\sqrt[5]{[Ca_3(PO_4)_2]^3}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | | | | | | |
| Y 11 | 6.81 | 1.95 | 100 | 100 | 2.80 | 1.70 | 1.10 | 2.84 | 5.97 | 25.22 | 7.3 | 0.206 | 0.455 | 0.035 |

*Preparation of Solution.*

21.1 cc. of 0.1 M $H_3PO_4$ were mixed with 134.75 cc. of 0.02 M $Ca(OH)_2$ 20 cc. of M $MgSO_4$ were added to this and the volume was made up to 200 cc. with distilled water. 2.58 equivalents of $Ca(OH)_2$ had thus been added. Solution was shaken continuously for 8 days at 38°C.

*Note.*—The error in this particular experiment is considerably greater than in the rest of our work, owing to the fact that the calcium in the solution could not be determined directly in the presence of such a high concentration of magnesium. The figure given for the calcium concentration is obtained from an analysis of the precipitate. The amount of calcium recovered from the precipitate was deducted from the total calcium introduced, and the difference was taken as the calcium in the solution. It is possible that the error in the calcium determination by this procedure is as great as 5 to 8 per cent.

For a salt of the type of $Ca_3(PO_4)_2$ this becomes:

$$\Delta pK_{s.p.} = 15\sqrt{\mu} \tag{35}$$

since $\nu = 5$, $\alpha' = 0.50$, and $z_1$ and $z_2$ are 2 and 3 respectively.

The value of $(pK_{s.p.})_\infty$ when $\Delta pK_{s.p.} = 0$, which is the solubility product at infinite dilution (and is identical with the solubility product expressed in terms of activities: $p^aK_{s.p.}$), is obtained by extrapolating the experimental solubility curves to zero ionic strength. From this extrapolation it appears that the solubility product for $Ca_3(PO_4)_2$ at infinite dilution has a value not fa · from $10^{-32.5}$ (or $pK_{s.p.} = 32.5$).

It is quite apparent from the curves in Fig. 8 that the solubility of $Ca_3(PO_4)_2$ remains a simple function of the ionic strength only in comparatively dilute solutions. When the ionic strength exceeds 0.01 $\mu$ (or $\sqrt{\mu}$ exceeds 0.1), marked specific ion effects come into play as is to be expected from the theory. Salts like KCl, NaCl, and $Na_2SO_4$ increase the solubility less than would be predicted from the simple relation, while on the other hand,



XUM

TABLE IX.

*Effect of the Inorganic Salts of Blood Serum on the Solubility of $Ca_3(PO_4)_2$ at 38°C.*

| Serial No. | Equivalents of $Ca(OH)_2$ added. | pH | $[Ca^{++}]$ | $[Mg^{++}]$ | $[Na^+]$ | $[K^+]$ | $[Cl^-]$ | $[P]$ | $[H_2PO_4^-]$ | $[HPO_4^{=}]$ | $[PO_4^{\equiv}]$ | Total $CO_2$ introduced. | Corrected $CO_2$ in solution. | $K_{s.p.}$ for $Ca_3(PO_4)_2 \times 10^{23}$. | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ $(32.5\text{-}pK_{s.p.})$. | $\mu$ | $\sqrt{\mu}$ | $f_{Ca_3(PO_4)_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mols* $\times 10^4$ | *mols* $\times 10^4$ | *mols* $\times 10^3$ | *mols* $\times 10^2$ | *mols* $\times 10^2$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^4$ | *mols* $\times 10^3$ | | | | | | |
| AA 1 | 1.20 | 7.02 | 1.43 | 6.0 | 130 | 6.0 | 107.2 | 6.47 | 3.24 | 3.39 | 1.42 | 3.00 | 2.86 | 5.83 | 27.23 | 5.3 | 0.133 | 0.365 | 0.087 |
| AA 2 | 1.25 | 6.95 | 1.25 | 6.0 | 130 | 6.0 | 107.2 | 6.31 | 3.34 | 2.97 | 1.06 | 3.00 | 2.86 | 2.19 | 27.66 | 4.8 | 0.132 | 0.363 | 0.110 |
| AA 3 | 1.30 | 7.05 | 1.50 | 6.0 | 130 | 6.0 | 107.2 | 6.08 | 2.87 | 3.21 | 1.44 | 3.00 | 2.88 | 7.04 | 27.15 | 5.35 | 0.132 | 0.363 | 0.085 |
| AA 4 | 1.40 | 7.05 | 1.23 | 6.0 | 130 | 6.0 | 107.2 | 5.86 | 2.76 | 3.10 | 1.39 | 3.00 | 2.85 | 3.55 | 27.45 | 5.05 | 0.132 | 0.363 | 0.098 |
| AA 5 | 1.28 | 6.92 | 1.45 | 5.85 | 127 | 5.85 | 105.0 | 7.51 | 4.10 | 3.41 | 1.135 | 2.93 | 2.77 | 3.92 | 27.41 | 5.1 | 0.130 | 0.361 | 0.096 |
| AA 6 | 1.80 | 7.22 | 1.53 | 6.0 | 130 | 6.0 | 107.2 | 4.51 | 1.69 | 2.81 | 1.87 | 3.00 | 2.87 | 12.3 | 26.91 | 5.6 | 0.131 | 0.362 | 0.076 |
| AA 7 | 2.00 | 7.22 | 1.33 | 6.0 | 130 | 6.0 | 107.2 | 3.91 | 1.47 | 2.44 | 1.62 | 3.00 | 2.85 | 6.09 | 27.22 | 5.3 | 0.130 | 0.361 | 0.087 |
| AA 8 | 2.20 | 7.29 | 0.95 | 6.0 | 130 | 6.0 | 107.2 | 3.20 | 1.025 | 2.12 | 1.65 | 3.00 | 2.85 | 2.33 | 27.63 | 4.9 | 0.129 | 0.359 | 0.105 |
| AA 9 | 2.40 | 7.40 | 1.25 | 6.0 | 130 | 6.0 | 107.2 | 2.63 | 0.75 | 1.88 | 1.89 | 3.00 | 2.84 | 7.98 | 27.10 | 5.4 | 0.128 | 0.358 | 0.083 |
| AA 10 | 2.08 | 7.10 | 1.53 | 5.85 | 127 | 5.85 | 105.0 | 4.41 | 1.95 | 2.46 | 1.24 | 2.92 | 2.75 | 5.43 | 27.27 | 5.2 | 0.127 | 0.357 | 0.091 |
| AA 11 | 2.80 | 7.70 | 1.15 | 6.0 | 130 | 6.0 | 107.2 | 1.63 | 0.27 | 1.36 | 2.715 | 3.00 | 2.75 | 11.2 | 26.95 | 5.55 | 0.127 | 0.356 | 0.078 |

Average $pK_{s.p.} = 27.21$.

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## CORRECTIONS.

On page 542, Vol. LXIV, No. 3, July, 1925, Table IX, below the column headings "Total $CO_3$ introduced" and "Corrected $CO_3$ in solution," read *mols* $\times 10^3$ for *mols* $\times 10^2$. The figures on the accompanying slip are to be pasted over those in Columns 18 and 19.

| $\mu$ | $\sqrt{\mu}$ |
|---|---|
| 0.146 | 0 382 |
| 0.145 | 0 381 |
| 0.145 | 0 381 |
| 0.145 | 0 381 |
| 0.141 | 0.376 |
| 0.144 | 0.380 |
| 0.143 | 0 378 |
| 0.142 | 0.377 |
| 0.141 | 0.376 |
| 0.140 | 0.374 |
| 0.140 | 0.374 |

Solutions were p
salt solutions were t
The final volume
M $H_2PO_4$ were adde
All solutions wer
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pH determinatior
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* Analyses of the
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$CaCO_3$ in the preci
solutions. This wa

TABLE IX.

*Effect of the Inorganic Salts of Blood Serum on the Solubility of $Ca_3(PO_4)_2$ at 38°C.*

| Serial No. | Equivalents of $Ca(OH)_2$ added. | pH | $[Ca^{++}]$ | $[Mg^{++}]$ | $[Na^+]$ | $[K^+]$ | $[Cl^-]$ | $[P]$ | $[H_2PO_4^-]$ | $[HPO_4^{=}]$ | $[PO_4^{\equiv}]$ | Total $CO_2$ introduced. | Corrected $CO_2$ in solution. | $K_{s.p.}$ for $Ca_3(PO_4)_2 \times 10^{25}$. | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ (32.5-$pK_{s.p.}$). | $\mu$ | $\sqrt{\mu}$ | $f_{Ca_3(PO_4)_2}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mols* $\times 10^4$ | *mols* $\times 10^4$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^2$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^4$ | *mols* $\times 10^3$ | | | | | | |
| AA 1 | 1.20 | 7.02 | 1.43 | 6.0 | 130 | 6.0 | 107.2 | 6.47 | 3.24 | 3.39 | 1.42 | 3.00 | 2.86 | 5.83 | 27.23 | 5.3 | 0.133 | 0.365 | 0.087 |
| AA 2 | 1.25 | 6.95 | 1.25 | 6.0 | 130 | 6.0 | 107.2 | 6.31 | 3.34 | 2.97 | 1.06 | 3.00 | 2.86 | 2.19 | 27.66 | 4.8 | 0.132 | 0.363 | 0.110 |
| AA 3 | 1.30 | 7.05 | 1.50 | 6.0 | 130 | 6.0 | 107.2 | 6.08 | 2.87 | 3.21 | 1.44 | 3.00 | 2.88 | 7.04 | 27.15 | 5.35 | 0.132 | 0.363 | 0.085 |
| AA 4 | 1.40 | 7.05 | 1.23 | 6.0 | 130 | 6.0 | 107.2 | 5.86 | 2.76 | 3.10 | 1.39 | 3.00 | 2.85 | 3.55 | 27.45 | 5.05 | 0.132 | 0.363 | 0.098 |
| AA 5 | 1.28 | 6.92 | 1.45 | 5.85 | 127 | 5.85 | 105.0 | 7.51 | 4.10 | 3.41 | 1.135 | 2.93 | 2.77 | 3.92 | 27.41 | 5.1 | 0.130 | 0.361 | 0.096 |
| AA 6 | 1.80 | 7.22 | 1.53 | 6.0 | 130 | 6.0 | 107.2 | 4.51 | 1.69 | 2.81 | 1.87 | 3.00 | 2.87 | 12.3 | 26.91 | 5.6 | 0.131 | 0.362 | 0.076 |
| AA 7 | 2.00 | 7.22 | 1.33 | 6.0 | 130 | 6.0 | 107.2 | 3.91 | 1.47 | 2.44 | 1.62 | 3.00 | 2.85 | 6.09 | 27.22 | 5.3 | 0.130 | 0.361 | 0.087 |
| AA 8 | 2.20 | 7.29 | 0.95 | 6.0 | 130 | 6.0 | 107.2 | 3.20 | 1.025 | 2.12 | 1.65 | 3.00 | 2.85 | 2.33 | 27.63 | 4.9 | 0.129 | 0.359 | 0.105 |
| AA 9 | 2.40 | 7.40 | 1.25 | 6.0 | 130 | 6.0 | 107.2 | 2.63 | 0.75 | 1.88 | 1.89 | 3.00 | 2.84 | 7.98 | 27.10 | 5.4 | 0.128 | 0.358 | 0.083 |
| AA 10 | 2.08 | 7.10 | 1.53 | 5.85 | 127 | 5.85 | 105.0 | 4.41 | 1.95 | 2.46 | 1.24 | 2.92 | 2.75 | 5.43 | 27.27 | 5.2 | 0.127 | 0.357 | 0.091 |
| AA 11 | 2.80 | 7.70 | 1.15 | 6.0 | 130 | 6.0 | 107.2 | 1.63 | 0.27 | 1.36 | 2.715 | 3.00 | 2.75 | 11.2 | 26.95 | 5.55 | 0.127 | 0.356 | 0.078 |

Average $pK_{s.p.} = 27.21$.

## CORRECTIONS.

On page 542, Vol. LXIV, No. 3, July, 1925, Table IX, below the column headings "Total $CO_2$ introduced" and "Corrected $CO_2$ in solution," read *mols* $\times 10^2$ for *mols* $\times 10^3$. The figures on the accompanying slip are to be pasted over those in Columns 18 and 19.

| $\mu$ | $\sqrt{\mu}$ |
|---|---|
| 0.146 | 0 382 |
| 0.145 | 0 381 |
| 0.145 | 0 381 |
| 0.145 | 0 381 |
| 0.141 | 0.376 |
| 0.144 | 0.380 |
| 0.143 | 0 378 |
| 0.142 | 0.377 |
| 0.141 | 0.376 |
| 0.140 | 0.374 |
| 0.140 | 0.374 |

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*Details of Experiment.*

Solutions were prepared by adding varying amounts of 0.02 M $Ca(OH)_2$ to 19.6 cc. of 0.1 M $H_3PO_4$. The following salt solutions were then added: 20 cc. of M NaCl; 1.2 cc. of M KCl; 6.0 cc. of M $NaHCO_3$; 4.8 cc. of 0.025 M $MgCl_2$.

The final volume was made up to 200 cc. with distilled water. In the case of Solutions AA5 and AA 10 24.5 cc. of 0.1 M $H_3PO_4$ were added instead of 19.6 cc. The final volume in these solutions was made up to 205 cc. instead of 200 cc.

All solutions were covered with a layer of albolene oil, sufficient to cover the solution completely even during agitation. Solutions were shaken continuously for 8 days at 38°C.

pH determinations were made colorimetrically by means of buffer solutions which had been checked electrometrically.

* Analyses of the precipitates in this series showed a Ca:P ratio always greater than 1.94. Ratios obtained varied between 2.05 and 2.20. It seemed likely that the excess of Ca was due to the presence of a small amount of $CaCO_3$ in the precipitate. We made no analyses for carbonate to verify this, but, on the assumption that the excess Ca in the precipitate was due to $CaCO_3$, we have deducted from the total carbonate introduced, an amount corresponding to the $CaCO_3$ in the precipitate. The residual $CO_3$ is the value given in the column above as the "corrected" $CO_3$ of the solutions. This was assumed to be entirely ionized as $HCO_3^-$.

$Ca(H_2PO_4)_2$ and $ZnSO_4$** increase the solubility more than would be expected. These specific effects, which are apparently related to the size and hydration of ions, as mentioned above, may be corrected for by using the more accurate formula:

$$\frac{\Delta pK_{s.p.}}{\nu} = \alpha' z_1 z_2 \sqrt{\mu} + \beta\mu \tag{36}$$

When the ionic strength of the solution is corrected by the intro-

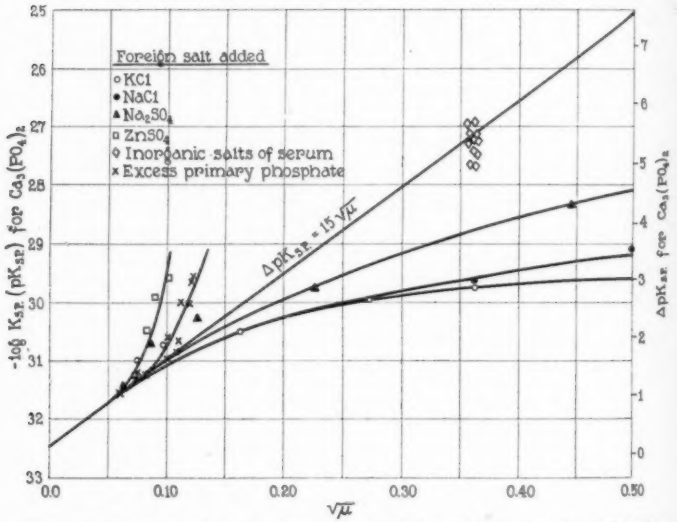


FIG. 8. Influence of the ionic strength of the solution on the solubility of $Ca_3(PO_4)_2$.

** The highly abnormal effects obtained with $Zn^{++}$ and $H_2PO_4^-$ ions cannot be explained on the basis of ionic size alone. Larger concentrations of $Zn^{++}$ produce a solid phosphate of zinc as mentioned previously, and it is very likely that it is this tendency to form a new compound which causes solubilities greater than those predicted by a purely physical theory as that of Debye and Hückel. The specific effect of $H_2PO_4^-$ may be due to the tendency to form $Ca(H_2PO_4)_2$. It would carry us too far afield to discuss this point further, but we wish to note that Linderstrøm-Lang (Linderstrøm-Lang, K., *Compt. rend. trav. Lab. Carlsberg*, 1924, xv, No. 4) uses the same explanation in his work on the salting-out effect on neutral molecules.

duction of such empirical $\beta$ coefficients,[17] it is found that the solubility changes are quite uniform. The solubility remains a linear function of the (corrected) ionic strength even in comparatively concentrated solutions, quite irrespective of the nature of the individual salts in the solution. This is shown clearly in Fig. 9 where solubility products (expressed as $pK_{s.p.}$ or $\Delta pK_{s.p.}$) are plotted against the "corrected ionic strength" of the solution: $\sqrt{\mu} + \beta\mu$.

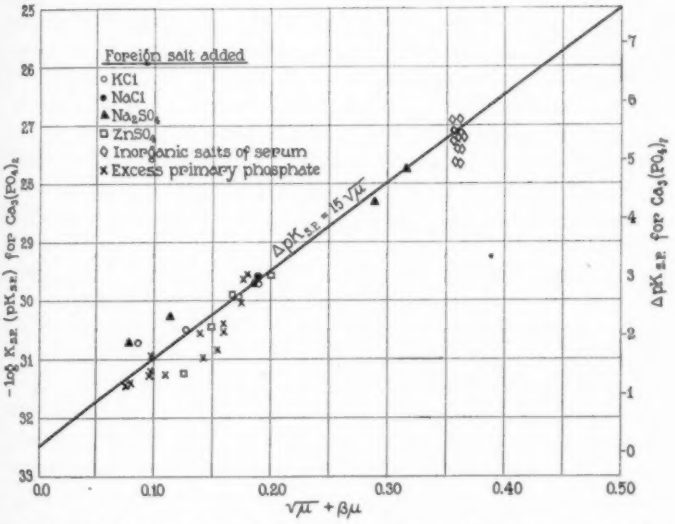


FIG. 9. Solubility of $Ca_3(PO_4)_2$ as a function of the "corrected" ionic strength of the solution.

[17] When equation (36) is transformed, it becomes:

$$\Delta\ pK_{s.p.} = \nu\ \alpha' z_1 z_2 \sqrt{\mu} + \nu\beta\mu$$

The linear correction term, strictly speaking, is, therefore, $\nu\beta\mu$, in this case $5\beta\mu$. Our values of $\beta$ contain the factor 5 and are, therefore, five times as great as the $\beta$ coefficients of Brönsted and others who have not incorporated the constant $\nu$ in their $\beta$ coefficients. It is, however, immaterial whether we call our constant $\beta$ or $5\beta$ since we are here interested in showing merely that the deviation from the simple theory is linear over a considerable range of concentration.

We have not attempted to evaluate $\beta$ coefficients for systems containing more than one foreign salt; under these conditions complications ensue. We have, however, plotted our observations on the system containing the inorganic salts of blood serum, for the reason that the solubility change produced by these salts is accounted[18] for by the simple formula, equation (35), without introducing any $\beta$ coefficient.

This latter series shows clearly that when a constant composition of foreign salts is maintained, as is the case in many biological systems, the stoichiometric solubility product remains constant. The importance of this constancy of the stoichiometric product will be discussed later.

Our data show that the theory of Debye and Hückel may be extended to account for the marked influence of salts on the solubility of a di-trivalent salt, $Ca_3(PO_4)_2$. In dilute solutions the simplified form of the Debye-Hückel equation, as used by Brönsted and La Mer, can be used to account for the observed changes, and the solubility then becomes a simple function of the ionic strength of the solution. When the solutions are more concentrated, specific ion effects are encountered. These specific effects may be accounted for by the introduction of a correction term which corrects theoretically for the size and hydration of the individual ions. The agreement of the data with the theory under these conditions is as good as can be expected in the case of an insoluble salt of unsymmetrical valence type, and especially when one considers the difficulties which are involved in measuring the $Ca_3(PO_4)_2$ system.

## X.

### *Solubility Product of $Ca_3(PO_4)_2$ in Blood Serum.*

In determining the solubility product for $Ca_3(PO_4)_2$ in blood serum, we encountered a number of difficulties. The temperature at which we worked (38°C.) is ideal for the growth of bacteria, and the resulting acidity and splitting of the proteins alter the character of the medium. To avoid this, we used commercial

[18] The variations in this series are due largely to the fact that the hydrogen ion concentrations were determined colorimetrically and the individual values are not as accurate as in the other determinations.

preparations of horse serum[19] to which a preservative (tricresol) had been added. Under these conditions the bacterial growth was very slight, even after 1 week of constant shaking.

In order to test the constancy of the stoichiometric product under varying concentrations of calcium and phosphate, we used the following experimental procedure: 190 cc. of serum were mixed with 10 cc. of a solution containing variable amounts of calcium and phosphate, and, in addition, precipitated $Ca_3(PO_4)_2$. Sufficient albolene oil was added to cover the surface of the liquid completely, even during agitation, and continuous shaking was then carried on for 1 week. The results of these experiments are given in Table X.

TABLE X.

*Solubility of $Ca_3(PO_4)_2$ in Horse Serum at 38°C.*

| Serial No. | pH | $[Ca^{++}]$ | [P] | $[PO_4^{\equiv}]$ | $K_{s.p.}$ for $Ca_3(PO_4)_2 \times 10^{-6}$ | $pK_{s.p.}$ | $\Delta pK_{s.p.}/(32.5-pK_{s.p.})$ | $\sqrt{Ca_3(PO_4)_2}$ |
|---|---|---|---|---|---|---|---|---|
| | | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^{10}$ | | | | |
| BB 1 | 6.75 | 2.10 | 1.71 | 13.8 | 1.77 | 25.75 | 6.75 | 0.045 |
| BB 2 | 6.85 | 1.785 | 1.19 | 14.0 | 1.11 | 25.96 | 6.5 | 0.050 |
| BB 3 | 6.80 | 1.97 | 0.936 | 9.13 | 0.637 | 26.20 | 6.3 | 0.055 |
| BB 4 | 6.80 | 2.06 | 0.774 | 7.55 | 0.497 | 26.30 | 6.2 | 0.058 |
| BB 5 | 6.80 | 2.89 | 0.710 | 6.93 | 1.16 | 25.94 | 6.6 | 0.048 |

The figures given indicate that the solubility product in blood serum is about $1 \times 10^{-21}$ ($pK_{s.p.} = 26.0$). The product remains nearly constant in spite of the variations in the calcium and phosphate contents of the serum.

When one compares the value of the $K_{s.p.}$ in blood serum with that obtained in a medium containing only the inorganic salts of blood serum, it might seem at first glance somewhat surprising that the increase in solubility in serum is no greater. The concentration of protein in the serum is high (7 to 8 per cent) and the valence of protein ions is so large that one might expect a much more striking difference. On the other hand, although the total quantity of protein present is great, the molecular weights of the

[19] Supplied through the courtesy of the New York State Health Department, Albany, N. Y.

serum proteins are so high (from 45,000 to 80,000) that the actual molar concentration of protein must be very small (probably less than 0.001 M). It is consequently not so surprising that the increased solubility in serum is no greater.

We have made no calculations of the ionic strength of serum, for the accuracy with which the molecular weights and valences of the serum proteins are at present known does not justify such calculations.

The solubility determinations on blood serum described above were undeniably crude. We therefore attempted to eliminate

TABLE XI.

*Solubility of $Ca_3(PO_4)_2$ in Blood Serum at 38°C.*

| Subject. | Length of time shaken. | pH | $[Ca^{++}]$ | [P] | $[PO_4^{\equiv}]$ | $K_{s.p.} \times 10^{26}$. | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ $(32.5-pK_{s.p.})$. | $\sqrt{Ca_3(PO_4)_2}$ |
|---|---|---|---|---|---|---|---|---|---|
| | wks. | | *mols* $\times 10^4$ | *mols* $\times 10^4$ | *mols* $\times 10^9$ | | | | |
| Horse. | | | | | | | | | |
| 5515-H | 2 | 7.40 | 6.75 | 6.77 | 4.90 | 0.738 | 26.13 | 6.4 | 0.052 |
| Human. | | | | | | | | | |
| T. C. G. | 1 | 7.70 | 4.50 | 4.84 | 8.08 | 0.594 | 26.23 | 6.3 | 0.055 |
| Human. | | | | | | | | | |
| C. O'D | 1 | 7.70 | 6.25 | 4.68 | 7.82 | 1.49 | 25.83 | 6.7 | 0.046 |

the possible sources of error (change in reaction, bacterial growth, presence of preservative, dilution of serum, etc.) by more refined experiments carried out on fresh serum under sterile conditions. Flasks containing solid $Ca_3(PO_4)_2$ and a suitable amount of albolene (to prevent the subsequent escape of $CO_2$) were sterilized, and freshly collected blood serum was introduced into these, using sterile technique. The flasks were then shaken continuously for 1 or 2 weeks. Horse serum[20] was used in some instances and serum from normal human adults in others. While many of these experiments proved failures on account of bacterial contamination, we were able to secure data on three specimens of serum where no such contamination occurred. These more accurate results,

[20] Horse serum was obtained from the H. K. Mulford Laboratories, Glenolden, Pa., through the courtesy of Dr. J. Reichel.

which are presented in Table XI, confirm the crude preliminary experiments described above. *The stoichiometric solubility product of $Ca_3(PO_4)_2$ in blood serum is found to be about $1 \times 10^{-26}$ ($pK_{s.p.} = 26.0$).*

XI.

*Solubility Product of $CaHPO_4$ in Salt Solutions.*

It was pointed out above that when $H_3PO_4$ is titrated with $Ca(OH)_2$ a certain region is found where the solid phase at equilibrium is secondary calcium phosphate, $CaHPO_4 \cdot 2H_2O$. This

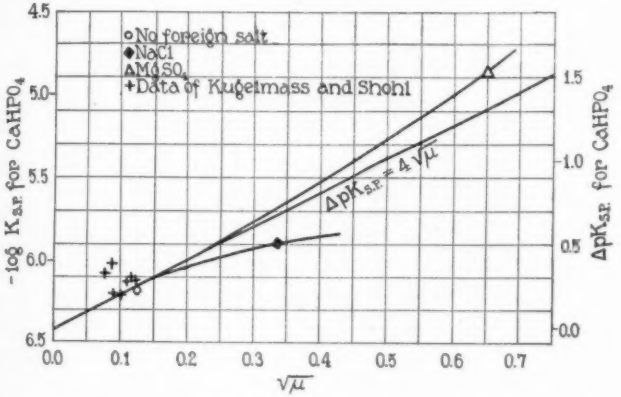


FIG. 10. Influence of the ionic strength of the solution on the solubility of $CaHPO_4$.

occurs in the absence of foreign salts, as well as when the titration is carried out in the presence of 0.1 M NaCl or 0.1 M $MgSO_4$. It is quite obvious that data from such equilibria can be used to calculate the solubility product constant for $CaHPO_4$ and to ascertain the effect which these two salts exert upon it.

In Table XII are given the data which we obtained on systems in equilibrium with $CaHPO_4$. As would be expected, the solubility of $CaHPO_4$, as measured by the stoichiometric solubility product, is somewhat increased in the presence of 0.1 M NaCl, and more markedly increased in 0.1 M $MgSO_4$. The effect of these foreign salts is, however, not nearly so great as in the case of ter-

TABLE XII.

*Effect of Foreign Salts on Solubility of $CaHPO_4$ at 38°C.*

| Serial No. | Equivalents of $Ca(OH)_2$ added. | pH | $[Ca^{++}]$ | $[Na^+]$ | $[Mg^{++}]$ | [P] | $[H_2PO_4^-]$ | $[HPO_4^{--}]$ | $[Cl^-]$ | $[SO_4^{--}]$ | $K_{s.p.} \times 10^7$. | $pK_{s.p.}$ | $\Delta pK_{s.p.}$ $(6.4-pK_{s.p.})$. | $\mu$ | $\sqrt{\mu}$ | $\frac{\sqrt{\mu}}{1+\beta\mu}$* | $\int CaHPO_4$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | *mols* $\times 10^3$ | | | | | | | |
| S 2 | 1.09 | 5.08 | 5.70 | | | 10.0 | 9.9 | 0.119 | | | 6.78 | 6.17 | 0.23 | 0.017 | 0.13 | | 0.77 |
| M 4 | 1.09 | 5.06 | 5.30 | | | 10.65 | 10.5 | 0.121 | | | 6.41 | 6.19 | 0.21 | 0.016 | 0.13 | | 0.79 |
| U 9 | 1.15 | 5.39 | 5.74 | 100 | | 9.53 | 9.3 | 0.228 | 100 | | 13.1 | 5.88 | 0.52 | 0.117 | 0.34 | 0.25 | 0.55 |
| U 10 | 1.18 | 5.36 | 5.45 | 100 | | 9.14 | 8.9 | 0.202 | 100 | | 11.0 | 5.96 | 0.44 | 0.116 | 0.34 | 0.25 | 0.60 |
| Y 4 | 1.67 | 6.28 | 8.31 | | 100 | 9.98 | 8.4 | 1.60 | | 100 | 133 | 4.88 | 1.52 | 0.424 | 0.65 | 0.76 | 0.174 |
| Y 6 | 1.73 | 6.305 | 8.50 | | 100 | 9.85 | 8.2 | 1.65 | | 100 | 140 | 4.85 | 1.55 | 0.422 | 0.65 | 0.76 | 0.168 |

*Preparation of Solutions.*

*Series S 2* .—20.62 cc. of 0.1 M $H_3PO_4$ were mixed with 56.2 cc. of 0.02 M $Ca(OH)_2$. Volume was made up to 200 cc. with distilled water. Shaken continuously for 9 days at 38°C.

*Series M 4*.—21.6 cc. of 0.1 M $H_3PO_4$ were mixed with 58.86 cc. of 0.02 M Ca $(OH)_2$. Volume made up to 200 cc. with distilled water. Shaken occasionally for 6 months, then shaken continuously 2 months. Temperature 38°C. Sterile technique followed.

*Series U*.—20.7 cc. of 0.1 M $H_3PO_4$ were mixed with appropriate amounts of 0.02 M $Ca(OH)_2$. 20 cc. of M NaCl were added, and the volume was made up to 200 cc. with distilled water. Shaken continuously for 8 days at 38°C.

*Series Y*.—21.1 cc. of 0.1 M $H_3PO_4$ were mixed with appropriate amounts of 0.02 M $Ca(OH)_2$ to give the desired number of equivalents. 20 cc. of M $MgSO_4$ were added, and the volume was made up to 200 cc. with distilled water. Continuous shaking for 8 days at 38°C.

* Value of $\beta$ taken as −0.77 for NaCl, and as 0.26 for $MgSO_4$.

XUM

tiary calcium phosphate,[21] as might be predicted from the fact that the secondary phosphate is a saturating salt of lower valence type.

In Fig. 10 the changes in solubility of $CaHPO_4$ are plotted as a function of the ionic strength of the solution. The ordinates represent stoichiometric solubility products of $CaHPO_4$, expressed in negative logarithms ($pK_{s.p.}$), and the abscissa units are square roots of the ionic strength ($\sqrt{\mu}$).

The straight line running diagonally across the figure represents the increase in solubility with ionic strength, calculated from the equation of Debye and Hückel, as simplified by Brönsted and La Mer:

$$\frac{\Delta pK_{s.p.}}{\nu} = \alpha' z_1 z_2 \sqrt{\mu} \tag{34}$$

For this particular case the equation becomes:

$$\Delta pK_{s.p.} = 4\sqrt{\mu} \tag{37}$$

since $\nu = 2$, $\alpha' = 0.50$, and $z_1$ and $z_2$ are both 2.

The value of $pK_{s.p.}$ when $\Delta pK_{s.p.} = 0$, in other words, the value of the solubility product at infinite dilution is obtained by extrapolating the experimental solubility curves to zero ionic strength, from which it appears that $pK_{s.p.}$ at infinite dilution, *or* $pK_{s.p.}$ *in terms of activities* ($p^aK_{s.p.}$) *is about 6.4 for* $CaHPO_4$ *at 38°C.*

It is evident from the figure that the changes in solubility cannot be expressed as a simple function of the ionic strength ($\sqrt{\mu}$) when the solutions are too concentrated[22] ($\sqrt{\mu}$ is greater than 0.15). The solubility of $CaHPO_4$ in 0.1 M $MgSO_4$ is somewhat greater than would be the case if this simple relation were universally applicable, while on the other hand, the solubility in 0.1 M NaCl is somewhat smaller. While these deviations could easily be

[21] 0.1 M NaCl increases the molar solubility of $Ca_3(PO_4)_2$ from $5.6 \times 10^{-7}$ to $13 \times 10^{-7}$, an increase of 130 per cent; while, on the other hand, the molar solubility of $CaHPO_4$ is increased from 0.00081 to 0.00112—an increase of only 38 per cent. 0.1 M $MgSO_4$ increases the solubility of $Ca_3(PO_4)_2$ about 1,660 per cent, but produces an increase of only 356 per cent in the solubility of $CaHPO_4$.

[22] That deviations due to the size of ions should occur at about this concentration is to be expected from the theory. See Brönsted and La Mer (19), p. 562.

Continuous shaking for 8 days at 38°C.
* Value of $\beta$ taken as $-0.77$ for NaCl, and as 0.26 for $MgSO_4$.

XUM

corrected by introducing $\beta$ coefficients, as was done in the case of tertiary calcium phosphate, such a correction would have little significance until more data have been collected, since the present data for $MgSO_4$ and NaCl represent only single points on the solubility curves.

As far as we are aware, the only other data in the literature on the solubility product constant for $CaHPO_4$ are those of Kugelmass and Shohl (9). These authors studied a system quite analogous to that employed in our experiments without foreign salt, for, with the exception of a small amount of carbonate, it contained only phosphates. Kugelmass and Shohl attempted to determine the solubility product constant on the basis of the older conception of incomplete dissociation of strong electrolytes. Factors representing the supposed degree of dissociation ($\alpha$) of the salts concerned were introduced into the stoichiometric concentrations, and the solubility product was then expressed in terms of the concentrations of the ions which were supposed to follow the gas laws. We have pointed out elsewhere, however, that the conductivity measurements of Noyes, from which these dissociation factors are computed, are no longer considered an accurate guide to the activity of the ions concerned. We have therefore recalculated the data of these observers in terms of stoichiometric ion concentrations. The data are given in Table XIII, and the values of the solubility product so obtained are plotted as a function of the ionic strength of the solution ($\sqrt{\mu}$) in Fig. 10. It can be seen from the figure, that with only two exceptions (in all probability experimental errors), the recalculated data of Kugelmass and Shohl are in excellent agreement with our own data.

XII.

*Calcium Buffers.*

Kugelmass (33) has recently called attention to the mechanism by which the $Ca^{++}$ ion concentration is buffered in the presence of insoluble calcium salts like the carbonate or phosphate, and he has given an extensive mathematical analysis of this action. For instance, in a system containing $PO_4^{\equiv}$ ions and in equilibrium with solid $Ca_3(PO_4)_2$, $Ca^{++}$ ions added in the form of a salt ike $CaCl_2$, instead of increasing the $Ca^{++}$ ion concentration, react

TABLE XIII.

*Data of Kugelmass and Shohl* on the Solubility of $CaHPO_4$ at 38°C. (Recalculated).*

| Serial No. | pH | [Ca] | [Na] | [P] | $[H_2PO_4^-]$ | $[HPO_4^=]$ | Total $CO_2$ | $[HCO_3^-]$ | $K_{a.p.} \times 10^7$ | $pK_{a.p.}$ | $\Delta pK_{a.p.}$ $(6.4-pK_{a.p.})$ | $\mu$ | $\sqrt{\mu}$ | $f_{CaHPO_4}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *mols* $\times 10^6$ | *mols* $\times 10^6$ | *mols* $\times 10^6$ | *mols* $\times 10^6$ | *mols* $\times 10^6$ | *mols* $\times 10^6$ | *mols* $\times 10^6$ | | | | | | |
| KS 1 | 7.28 | 2.35 | | 0.64 | 0.220 | 0.42 | 4.5 | 3.8 | 9.87 | 6.01 | 0.39 | 0.0076 | 0.087 | 0.64 (?) |
| KS 2 | 7.24 | 2.62 | | 0.50 | 0.188 | 0.32 | 5.6 | 4.2 | 8.25 | 6.08 | 0.32 | 0.0062 | 0.079 | 0.69 (?) |
| KS 3 | 7.05 | 2.70 | | 0.46 | 0.217 | 0.24 | 6.3 | 4.8 | 6.56 | 6.18 | 0.22 | 0.0084 | 0.092 | 0.78 |
| KS 4 | 7.02 | 2.85 | | 0.44 | 0.211 | 0.23 | 7.0 | 5.3 | 6.53 | 6.19 | 0.21 | 0.0089 | 0.094 | 0.79 |
| KS 5 | 6.92 | 2.00 | 7.5 | 0.69 | 0.388 | 0.31 | 12.8 | 9.8 | 6.20 | 6.20 | 0.20 | 0.0110 | 0.105 | 0.79 |
| KS 6 | 6.92 | 2.15 | 7.5 | 0.81 | 0.442 | 0.37 | 13.1 | 9.3 | 7.91 | 6.10 | 0.30 | 0.0136 | 0.116 | 0.71 |
| KS 7 | 6.85 | 2.32 | 7.5 | 0.78 | 0.456 | 0.32 | 13.9 | 9.4 | 7.49 | 6.13 | 0.27 | 0.0134 | 0.115 | 0.73 |
| KS 8 | 6.80 | 2.50 | 7.5 | 0.64 | 0.392 | 0.25 | 15.2 | 9.9 | 6.26 | 6.20 | 0.20 | 0.0144 | 0.120 | 0.79 |

* See Kugelmass and Shohl (9), p. 658, Table III.

with $PO_4^{\equiv}$ ions to form more solid tertiary phosphate, with the result that very little change in the $Ca^{++}$ ion concentration occurs.

The curve showing the relative buffer effects of carbonates and phosphates on the $Ca^{++}$ ion concentration is given in Fig. 2 of Kugelmass' paper (33). The calculations of Kugelmass plotted in this graph show that the buffer effect of carbonates is always two-thirds as great as that of phosphates. That this must be true is evident, when one considers that 2 mols of phosphate can combine with 3 mols of calcium to form an insoluble salt, while on the other hand, 2 mols of carbonate can combine with only 2 mols of calcium to form the insoluble $CaCO_3$.††

According to Kugelmass there is an intimate relation between

†† Kugelmass has not evaluated the actual numerical value of the phosphates as calcion buffers for an obvious reason. In the equation which he proposes for that purpose ((33), p. 243, equation (10)):

$$pCa = pK'' + \frac{2}{3} \log \frac{[B_2HPO_4]^2}{[BH_2PO_4]}$$

the constant $K''$ is a combined constant composed in part of the $K_{s.p.}$ for $Ca_3(PO_4)_2$ which was at that time unknown. (Kugelmass' constant is really $\frac{K_2^2}{K_3^2} \cdot K_{s.p.}$ corrected for degrees of dissociation of the phosphates, where $K_2$ and $K_3$ are the secondary and tertiary ionization constants of phosphoric acid.) Since the value of the phosphates as calcion buffers depends on the solubility product constant of $Ca_3(PO_4)_2$ it is clear that this constant must be known before their buffer effect can be evaluated. Furthermore, as we have pointed out in the preceding part of this paper, the solubility product constant must be evaluated for each particular medium employed, owing to the marked salt and protein effects to which it is subject (or, what amounts to the same thing, the activity coefficients for the ions composing the salt must be known for each medium). Kugelmass does not employ ion activities, but attempts to obtain the same result by introducing the so called "degree of ionization" of the calcium salts obtained from conductivity data in other media. Such a procedure is open to serious objection in a thermodynamic treatment. Although this method has been used with fair success in the case of dilute uni-univalent electrolytes on the basis of the classical ionization theory, recent work (34) has shown that agreement is not found between the conductivity "degree of ionization" and the activity coefficient *even with uni-univalent electrolytes.* When the valence of the ions is higher, the discrepancies may amount to 100 per cent or even more.

the dissociation constant of the buffer acid and the pH at which the calcion buffer effect is exerted. Thus he calculates that the maximum buffer effect occurs at a hydrogen ion concentration where the value of $[H^+]$ is nearly identical with the value of the dissociation constant; with phosphates the maximum buffer effect should therefore occur at pH 7.0 ($[H^+] = 10^{-7}$). No experimental data are, however, presented to establish this point. The systems which we have studied in our titration curve differ from that discussed by Kugelmass in that we have added $Ca(OH)_2$ instead of a salt like $CaCl_2$. While it is possible that maximum buffer effects may occur at pH 7.0 when a salt like $CaCl_2$ is added, yet in the systems studied by us it is clear that such buffer effects occur at quite different hydrogen ion concentrations, the pH at which the buffer effect occurs depending upon the $K_{s.p.}$ of $Ca_3(PO_4)_2$ in the medium under consideration.

It has been pointed out previously that the plateau in the titration curves of $H_3PO_4$ with $Ca(OH)_2$ (see Figs. 1, 2, and 3) is due to a buffering of the hydrogen ion concentration owing to the formation of the precipitate $Ca_3(PO_4)_2$. Hydroxyl ions are used up in the rearrangement and consequently the pH remains almost constant. It is equally clear that since $Ca^{++}$ ions are being used up and converted into $Ca_3(PO_4)_2$ at the same time, it must be at this pH (*i.e.* the pH of the plateau, pH 5.0 at 38°C. in the absence of foreign salt) that a buffering of the $Ca^{++}$ concentration also occurs. Before the titration curve reaches the plateau there can be no $Ca^{++}$ buffer effect, because no precipitate has yet formed. After the plateau has been passed there can also be no $Ca^{++}$ buffer effect, because all the available $PO_4^{\equiv}$ has now been precipitated, consequently there is no mechanism for removing any additional $Ca^{++}$ introduced. Since at these two points the buffer effect may be considered zero, and in the region of the plateau (between them) there is a definite buffer effect, it is clear that the buffer effect must rise to a maximum and then decline, and that this maximum must occur at the pH of the plateau. In the curves given in Fig. 2, where titrations were carried out in the presence of 0.1 M NaCl and 0.1 M $MgSO_4$, the plateau occurs at a somewhat different pH (pH 5.6 for 0.1 M NaCl and pH 6.4 for 0.1 M $MgSO_4$). From this one may infer that the maximum calcium buffer effect occurs at these higher pH values when these

salts are present. Since the plateau changes with temperature, the pH at which the maximum $Ca^{++}$ buffer effect occurs must also be a function of the temperature. It is thus clear that calcium ions as well as hydrogen ions are buffered best at the pH of the plateau—a pH region which depends primarily on the $K_{s.p.}$ of $Ca_3(PO_4)_2$ in the medium under consideration, and only indirectly upon the ionization constants of $H_3PO_4$.

We have not attempted to evaluate the calcion buffer effect directly, inasmuch as a reliable, electrometric method for determining the activity of the calcium ion, $a_{Ca^{++}}$, is not yet available, nor can the data obtained from conductivity measurements be employed to evaluate this in mixtures with any degree of numerical accuracy. We have, however, studied the buffering of the stoichiometric calcium concentration which takes place in such systems. In Fig. 11*a* the stoichiometric $Ca^{++}$ is plotted as a function of additions of $Ca(OH)_2$. The data are taken from titrations of $H_3PO_4$ with $Ca(OH)_2$ carried out in 0.1 M $MgSO_4$, in 0.1 M NaCl, and in the absence of foreign salt. From these curves it can be seen that in the region where $Ca_3(PO_4)_2$ is being precipitated, an anomalous buffer action takes place: *the addition of $Ca^{++}$ in the form of $Ca(OH)_2$ actually decreases the calcium content of the solution.*

This anomaly is not so surprising when one considers the ionic reactions which are taking place. At the pH of our solution most of the phosphate exists in the form of $H_2PO_4^-$. When we add to this *dn* mols of $Ca(OH)_2$, the $2dn$ mols of $OH^-$ react quantitatively with the $H_2PO_4^-$ according to the equation:

$$H_2PO_4^- + 2OH^- \rightarrow PO_4^{\equiv} + 2H_2O \tag{38}$$

yielding an increase of *dn* mols of $PO_4^{\equiv}$. But, since $Ca^{++}$ ions are present, the additional *dn* mols of $PO_4^{\equiv}$ react with $Ca^{++}$ according to the equation:

$$1\tfrac{1}{2}Ca^{++} + PO_4^{\equiv} \rightarrow \tfrac{1}{2}Ca_3(PO_4)_2 \tag{39}$$

Thus the addition of *dn* mols of $Ca(OH)_2$ causes a loss of $1\frac{1}{2}$ *dn* mols of $Ca^{++}$ from the solution, whereas only *dn* mols of $Ca^{++}$ have been added. The net result is the loss of $\frac{1}{2}dn$ mols of $Ca^{++}$.

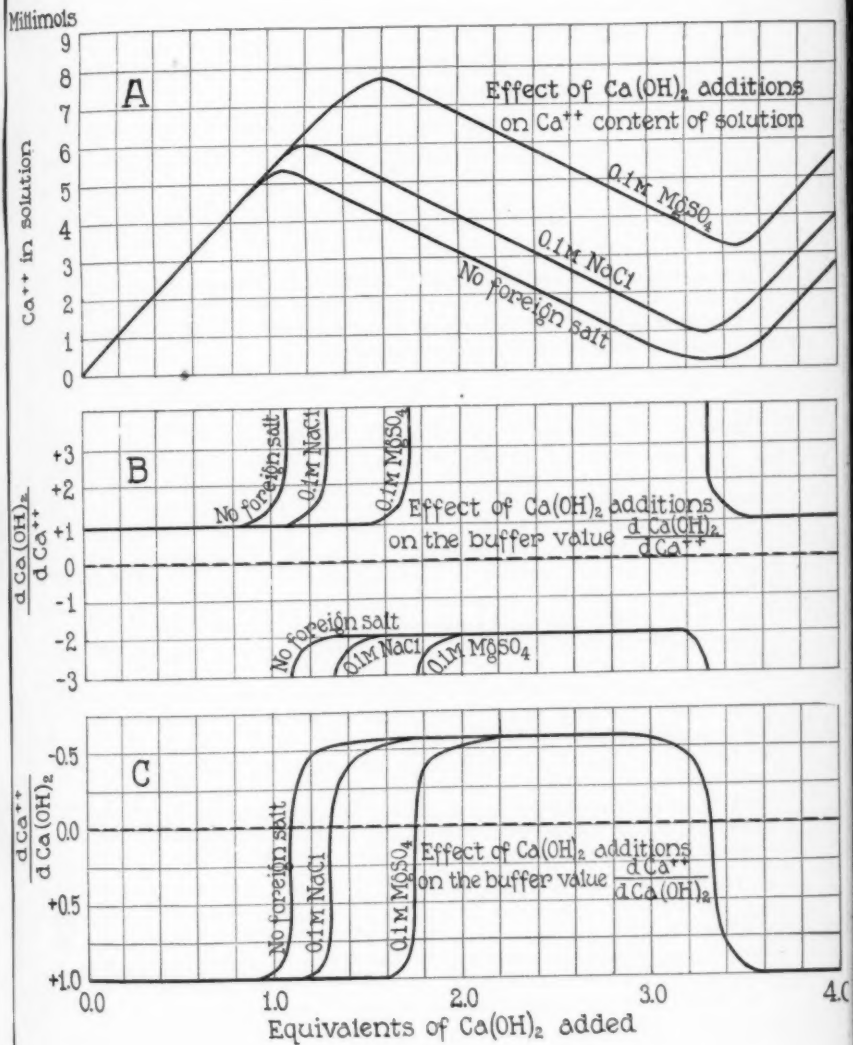
Millimols
A
Effect of Ca(OH)2 additions
on Ca++ content of solution
Ca++ in solution
0.1M MgSO4
0.1M NaCl
No foreign salt
B
Effect of Ca(OH)2 additions
on the buffer value d Ca(OH)2 / d Ca++
d Ca(OH)2 / d Ca++
No foreign salt
0.1M NaCl
0.1M MgSO4
C
Effect of Ca(OH)2 additions
on the buffer value d Ca++ / d Ca(OH)2
d Ca++ / d Ca(OH)2
No foreign salt
0.1M NaCl
0.1M MgSO4
Equivalents of Ca(OH)2 added

FIG. 11.

It is therefore clear why additions of $Ca(OH)_2$ actually decrease the $Ca^{++}$ concentration.[23]

As a unit for expressing such stoichiometric calcium buffer effects we have chosen the differential ratio:

$$\frac{d\,Ca^{++}_{(\text{in solution})}}{d\,Ca^{++}_{(\text{added})}}$$

which in this case becomes $\left(\frac{d\,Ca^{++}_{(\text{in solution})}}{d\,Ca(OH)_2}\right)$. This ratio,

[23] An analogous reaction is employed in the treatment of "temporary hardness" in water by the addition of slaked lime. The soluble bicarbonate of calcium to which the hardness is due is converted into the insoluble normal carbonate, according to the equation:

$$Ca^{++} + 2HCO_3^- + Ca^{++} + 2OH^- \rightarrow 2CaCO_3 \downarrow + 2H_2O$$

Thus the $Ca^{++}$ is reduced by the addition of $Ca(OH)_2$.

Whether or not this negative buffer effect which we have observed would occur if we were considering the activity of the calcium, instead of stoichiometric concentration, is somewhat doubtful. It seems likely that the greater part, if not all, of the negative slope of the stoichiometric $Ca^{++}$ curve may be due to the change in the activity coefficient of the calcium as the salt concentration of the medium is altered. This supposition is borne out by the titration curve of $H_3PO_4$ with $Ca(OH)_2$ in the presence of the inorganic salts of blood serum (Table IX). Under these conditions the change in the character of the medium is quite negligible and, hence, only a negligible change in the activity coefficient can take place. It is noteworthy that under this condition the stoichiometric $Ca^{++}$ concentration, which is then directly proportional to the activity of the $Ca^{++}$, remains practically constant.

On the other hand, it must be borne in mind that the activity coefficients which we have calculated in our tables refer to the activity of the salt, not to the individual activities of the $Ca^{++}$ or $PO_4^{\equiv}$ ions. We are, therefore, calculating the geometric mean of these two ion activity coefficients, namely:

$$f_{Ca_3(PO_4)_2} = \sqrt[5]{f^3_{Ca^{++}} \times f^3_{PO_4^{\equiv}}}$$

While it is quite possible that the individual ion activity coefficients vary proportionally with the change in character of the medium, it may be that specific ion effects occur which influence one activity coefficient quite differently from the other. On this account it seems to us premature to make a definite statement about the behavior of the activity coefficient or the activity of the $Ca^{++}$ ion, until this individual ion activity can be measured directly.

which we may designate as our buffer value, expresses the relationship between the increment of $Ca^{++}$ in the solution and the increment of $Ca^{++}$ added. It is immaterial what units are used in this expression, provided that the same units are used in the numerator and denominator. It follows from the above that our definition of calcium buffers is a somewhat broader one than that suggested by Kugelmass. Calcium buffers are substances which, in solution, resist changes in the $Ca^{++}$ concentration when $Ca^{++}$ ions are added *in any form.*

The advantage of using the differential ratio: $\frac{d\ Ca^{++}}{d\ Ca(OH)_2}$ in this particular form can be seen from an inspection of Figs. 11*b* and 11*c* where we have plotted[24] stoichiometric $Ca^{++}$ buffer effects against concentrations of $Ca(OH)_2$ added. When the buffer value is defined as $\frac{d\ Ca(OH)_2}{d\ Ca^{++}}$ it can be seen from the curve (Fig. 11*b*) that infinite buffer values occur at the points where the precipitation of $Ca_3(PO_4)_2$ commences or terminates. Discontinuous curves are therefore obtained. If we follow Kugelmass and define our buffer value for the present system as $\frac{d\ Ca(OH)_2}{d\ p\ Ca}$ the same difficulty remains. We have, therefore, chosen to define the buffer value as $\frac{d\ Ca^{++}}{d\ Ca(OH)_2}$. This procedure is more in accord with mathematical convention,[25] and has the advantage that continuous curves (Fig. 11*c*) are obtained even in the region of the anomalous negative buffer effect. *It should be noted that a decrease in the value of this expression denotes increased buffering. Thus, when* $\frac{d\ Ca^{++}}{d\ Ca(OH)_2}$ *is positive, the addition of* $Ca(OH)_2$ *produces an increase in the* $Ca^{++}$ *concentration; when the value of this expression is zero, no change in* $Ca^{++}$ *concentration occurs; when this value becomes negative, the addition of* $Ca(OH)_2$ *causes a decrease in the* $Ca^{++}$ *concentration.*

An inspection of the buffer curves in Fig. 11*c* reveals the follow-

[24] The diagrams given in Figs. 11 and 12 are somewhat schematic, and are intended merely to show the general nature of the buffer curves.

[25] Since $Ca(OH)_2$ is the independent variable and, conventionally, the independent variable is always placed in the denominator.

XUM

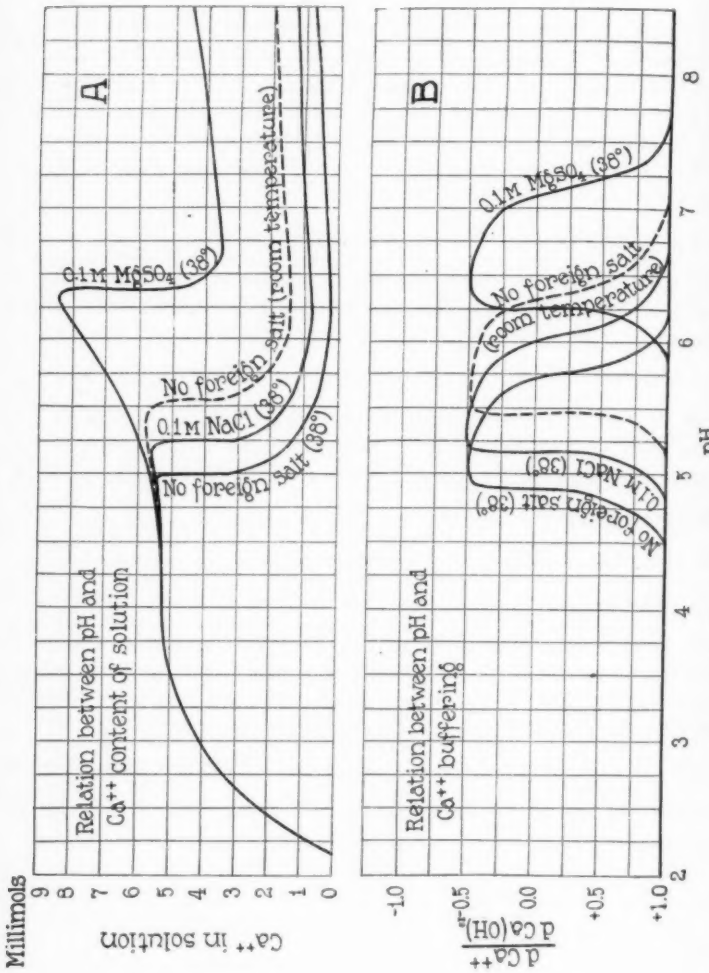
A
Relation between pH and Ca++ content of solution
Millimols
Ca++ in solution
0.1 M MgSO4 (38°)
No foreign salt (room temperature)
0.1 M NaCl (38°)
No foreign salt (38°)
B
Relation between pH and Ca++ buffering
d Ca++ / d Ca(OH)2
0.1 M MgSO4 (38°)
No foreign salt (room temperature)
0.1 M NaCl (38°)
No foreign salt (38°)
pH

FIG. 12.

ing facts. The slopes of the three buffer curves are practically identical, and the height to which each attains, which measures the maximum buffer effect, is also identical.

This latter fact is evident when one considers that the maximum buffer effect is determined in all cases by the valence of the $PO_4^{\equiv}$ ion and by the stoichiometric relations which we have discussed above (equations (38) and (39)). On the other hand, it is equally clear that the "total buffer effect" of the phosphoric acid on the $Ca^{++}$ concentration (in other words the total resistance offered by the $H_3PO_4$ to the increase of $Ca^{++}$ as $Ca(OH)_2$ was added) differs markedly in the three cases under consideration, depending upon the solubility of $Ca_3(PO_4)_2$ in each particular system. The area beneath the buffer curves measures this "total buffer effect." It can be seen from the diagram that the magnitude of this buffer effect is inversely proportional to the solubility of $Ca_3(PO_4)_2$—there is less buffering as $Ca_3(PO_4)_2$ becomes more soluble.[26]

The relation between pH and stoichiometric $Ca^{++}$ buffering is shown in Figs. 12*a* and 12*b*. In Fig. 12*a* the $Ca^{++}$ content of the solution, and in Fig. 12*b* the buffer value $\frac{d\ Ca^{++}}{d\ Ca(OH)_2}$ is plotted as a function of the pH of the solution. The four systems which we have plotted show the effect of factors like temperature and foreign salt concentrations, which alter the solubility of $Ca_3(PO_4)_2$. Although the extent of the buffer action, as measured on the pH scale, does not differ appreciably in the systems studied, it is quite clear that the *actual* pH at which the buffer effect occurs, and the pH at which the maximum buffer effect occurs, varies quite markedly. The pH region at which the buffer effect occurs seems to depend upon the solubility product of $Ca_3(PO_4)_2$. When $Ca_3(PO_4)_2$ is more soluble, the buffer effect occurs at a higher pH.

FIG. 12.

## XIII.

### *Solubility Relations of Phosphates in the Animal Organism.*

In the preceding part of this paper we have shown how the solubility product constants for $CaHPO_4$ and $Ca_3(PO_4)_2$ may be

[26] This is quite analogous to the changes in the total hydrogen ion buffer effect produced by alterations in the solubility of $Ca_3(PO_4)_2$, which was discussed on pages 514 and 525.

XUM

evaluated. Since the individual activities of the ions which compose these salts cannot be accurately measured, we have chosen the stoichiometric solubility product as the most convenient standard for reference. As we have pointed out, however, the stoichiometric product is constant only under conditions of constant salt concentration. It must therefore be evaluated for any particular biological medium in which such solubility relations are being considered. It remains to point out the part which these solubility relations play in various physiological processes.

As far as our present knowledge goes, it is doubtful whether the solubility of $CaHPO_4$ is of any biological importance. The presence of solid $CaHPO_4$ in bones and in pathological calcifications has yet to be demonstrated. Certainly it does not exist there in more than traces. There is no evidence that solid $CaHPO_4$ exists anywhere else in the body. On the other hand, it has been suggested (35) that $CaHPO_4$ occurs as an intermediate solid phase in the deposition of $Ca_3(PO_4)_2$ in bones and elsewhere; an equilibrium exists between $CaHPO_4$ and $CO_2$ according to the equation:

$$(40) \qquad 2CaHPO_4 + Ca^{++} + 2HCO_3^- \rightleftarrows 2H_2O + 2CO_2 + Ca_3(PO_4)_2$$

when the $CO_2$ tension is reduced the equilibrium swings to the right and $Ca_3(PO_4)_2$ is precipitated at the expense of $CaHPO_4$.

The question of the transitory existence of $CaHPO_4$ during the deposition of $Ca_3(PO_4)_2$ is more fully discussed in the following paper of this series. It is, however, quite evident that $CaHPO_4$ can play such an intermediate rôle *only when the conditions are such that at some time or other, the solubility product of $CaHPO_4$ is exceeded.* Until it has been demonstrated that this occurs in the body, one cannot attribute to $CaHPO_4$ even such an intermediate rôle.

No such doubt exists as to the biological importance of $Ca_3(PO_4)_2$. That this salt constitutes by far the greater part of the inorganic matter of bones, teeth, and many pathological deposits is well known, and it is clear that no quantitative studies of calcification in these tissues can be made without a knowledge of the solubility product of $Ca_3(PO_4)_2$. In the third paper of this series we have attempted to apply our data on the $K_{s.p.}$ of $Ca_3(PO_4)_2$ to the problem of bone calcification.

The relation between calcification and the solubility of $Ca_3(PO_4)_2$ is an obvious one. What is not so commonly appreciated, however, is the fact that the absorption of calcium and phosphorus by the body is directly dependent on the solubility of $Ca_3(PO_4)_2$. The intestinal contents of infants contain large amounts of $Ca_3(PO_4)_2$, and it is probable that this substance exists in the intestines of most adults—particularly when milk forms any considerable portion of the diet. There is much evidence[27] to indicate that solid $Ca_3(PO_4)_2$ cannot be absorbed by the body—in other words, only free $Ca^{++}$ ions or the various types of phosphate ions can pass through the wall of the intestine. The solubility product of $Ca_3(PO_4)_2$ thus remains the factor which limits the concentrations of ions available for absorption. Fortunately the reaction of the intestinal contents is usually on the acid side of neutrality, and the fraction of the total phosphorus ionized as $PO_4^{\equiv}$ is therefore so small that considerable concentrations of $Ca^{++}$, $H_2PO_4^-$, and $HPO_4^=$ ions can exist without exceeding the $K_{s.p.}$ of $Ca_3(PO_4)_2$. When the reaction becomes more alkaline, however, defective absorption ensues (37).

The excretion of calcium in urine seems to bear a relation to the solubility product of $Ca_3(PO_4)_2$. Large amounts of inorganic phosphate are regularly secreted by the kidneys, this being one of the mechanisms by which the acid-base equilibrium in the body is maintained. Although the amount of calcium excreted in the urine is usually very small, it has been noted that highly acid urines often contain more calcium, which suggests that we are here dealing with a solubility mechanism, and that only soluble material can be excreted by the kidney. On the other hand, it seems probable that other insoluble salts of calcium, like the carbonate, urate, and perhaps the oxalate, also play a limiting part here. The system is, moreover, complicated by the fact that the ionic strength of urine, and hence the solubility of the insoluble calcium salts in it, is subject to great variations.

The solubility relations of $Ca_3(PO_4)_2$ in the body are, however, not so simple as one might suppose. The phenomenon of delayed equilibrium is a factor of considerable importance. This will be taken up more fully in the following paper.

[27] Reviewed by some of us in a recent publication (36).

XIV.

SUMMARY.

1. A method is described for evaluating the solubility product constants of $CaHPO_4$ and $Ca_3(PO_4)_2$ from the titration data of $H_3PO_4$ with $Ca(OH)_2$. The pH ranges at which $CaHPO_4$ and $Ca_3(PO_4)_2$ are stable have been established.

2. The solubility product in terms of activities (*i.e.* the stoichiometric solubility product at infinite dilution) is found to be $10^{-6.4}$ for $CaHPO_4$ and $10^{-32.5}$ for $Ca_3(PO_4)_2$ at 38°C.

3. When expressed in stoichiometric units, it is found that these solubility product constants are enormously affected by the presence of other salts. These variations are found to be in agreement with the theory of solubility in salt solutions, as developed by Brönsted and by Debye and Hückel.

4. The stoichiometric solubility product constant for $Ca_3(PO_4)_2$ in blood serum is found to be $10^{-2}$; in a solution containing the inorganic salts of blood serum the value is about $10^{-27.2}$ at 38°C.

5. Certain anomalous calcium buffer effects are discussed.

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XUM

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# STUDIES IN CALCIFICATION.

## II. DELAYED EQUILIBRIUM BETWEEN THE CALCIUM PHOSPHATES AND ITS BIOLOGICAL SIGNIFICANCE.

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The extreme slowness with which certain solutions of calcium phosphates approach equilibrium has been noted by a number of observers (1–6). Thus in some of the systems studied (3, 4) evidence of incomplete equilibrium was still present after more than a year of continuous shaking at constant temperature. In the titration experiments of $H_3PO_4$ with $Ca(OH)_2$ described in the preceding paper this phenomenon was encountered throughout the range of solutions in which the final precipitate was found to be $Ca_3(PO_4)_2$. At least 10 days of continuous shaking were required before an approximate equilibrium was obtained.

As a possible explanation for the delay in reaching equilibrium Bassett[1] suggests that the crystalline salt $CaHPO_4$ is first precipitated. The more slowly precipitated $Ca_3(PO_4)_2$ or a more basic salt soon forms a gelatinous layer about these crystals, preventing free diffusion and thus impeding equilibrium formation. The resulting solid phase is a mixture of secondary and tertiary calcium phosphates and perhaps $Ca(OH)_2$. He found great difficulty in obtaining a precipitate with constant composition and concludes with others that (7) "die Zusammensetzung des Künstlich gefällten amorphen sogenannten Tricalciumphosphates im allgemeinen sehr weit von der Formel abweicht."

The results of our own experiments described in the preceding

[1] Bassett (4), p. 621.



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paper, however, indicate that after 10 days continuous shaking a solid phase is obtained which shows quite uniformly the Ca:P ratio characteristic of $Ca_3(PO_4)_2$.*

Wendt and Clarke (6) offer a somewhat similar explanation for the delay in equilibrium which they encountered in their titration experiments. According to these authors the first precipitate which appears is $CaHPO_4$. This, however, is unstable and decomposes into primary and tertiary phosphates according to the equation:

$$4\ CaHPO_4 \rightarrow Ca(H_2PO_4)_2 + Ca_3(PO_4)_2$$

yielding the soluble primary phosphate and leaving the insoluble tertiary phosphate as the final precipitate. No analyses were, however, made to support these conclusions.

The experiments reported in this paper were undertaken with the hope of throwing more light on the question of this delay in equilibrium. We undertook to confirm the transitory existence of solid $CaHPO_4$ under these conditions and to relate, if possible, its appearance and disappearance with the solubility product data obtained in the previous paper.

Preliminary experiments were undertaken to determine the effect of time on the composition of the solid phase. A solution of $H_3PO_4$ was prepared to which 1.20 equivalents of $Ca(OH)_2$ had been added. After occasional shaking for 24 hours the precipitate was removed, and on analysis was found to have a Ca:P ratio of 1.41.† A solution similarly prepared was shaken continuously for 10 days. At the end of this period the precipitate was found to have a Ca:P ratio of 1.96. The same experiment was performed in a solution of $H_3PO_4$ to which 2.13 equivalents of $Ca(OH)_2$ had been added. The solution was shaken occasionally, and 24 hours after mixing, the precipitate was removed, and on analysis was found to have a Ca:P ratio of 1.81. A similarly prepared solution, which was shaken continuously for 10 days, was found at the end of that time to have yielded a precipitate in which the Ca:P ratio was 1.93.

* It is possible that the extreme delay noted by Bassett was due to the fact that he used solid CaO or solid phosphates of calcium as the initial substances in his experiments, while we have uniformly used clear filtered lime water.

† The theoretical Ca:P ratio for $Ca_3(PO_4)_2$ is 1.94; for $CaHPO_4$ it is 1.29.

These experiments, which were carried out at 38°C., indicate clearly the transitory existence of the solid phase $CaHPO_4$. We then attempted to study the ionic equilibria in such a solution from day to day, and to determine the relation which existed between the ion products for secondary and tertiary calcium phosphates and the solubility product constants for these salts.

A solution of $H_3PO_4$ was prepared to which 2.13 equivalents of $Ca(OH)_2$ had been added. This was kept in a thermostat at 38°C. and was shaken once daily. Samples of the solution were

TABLE I.

*Effect of Time on Stoichiometric Ion Products of $CaHPO_4$ and $Ca_3(PO_4)_2$ at 38°C.*

| Time after mixing. | pH | Calcium. | | Phosphorus. | | $[HPO_4^{=}]$ | $[PO_4^{\equiv}]$ | $[Ca^{++}] \times [HPO_4^{=}] \times 10^7$ | $-$ log product for $CaHPO_4$. | $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2 \times 10^{29}$ | $-$ log product for $Ca_3(PO_4)_2$. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *hrs.* | | *mg. per 100 cc.* | *mols* $\times 10^3$ | *mg. per 100 cc.* | *mols* $\times 10^3$ | *mols* $\times 10^4$ | *mols* $\times 10^{12}$ | | | | |
| 24 | 5.63 | 9.4 | 2.35 | 14.1 | 4.56 | 2.23 | 31.8 | 5.24 | 6.28 | 13.1 | 28.88 |
| *days* | | | | | | | | | | | |
| 2 | 5.52 | 10.1 | 2.52 | 15.5 | 4.99 | 1.62 | 21.2 | 4.09 | 6.39 | 7.24 | 29.14 |
| 3 | 5.41 | 10.5 | 2.62 | 17.0 | 5.48 | 1.37 | 14.1 | 3.59 | 6.445 | 3.58 | 29.45 |
| 4 | 5.33 | 10.0 | 2.50 | 15.4 | 4.97 | 1.04 | 8.9 | 2.60 | 6.585 | 1.23 | 29.91 |
| 6 | 5.19 | 9.8 | 2.45 | 14.1 | 4.56 | 0.69 | 4.3 | 1.70 | 6.77 | 0.272 | 30.57 |
| 9 | 5.06 | 10.0 | 2.50 | 16.7 | 5.38 | 0.57 | 2.8 | 1.44 | 6.84 | 0.124 | 30.91 |
| ∞ | 4.955 | 10.9 | 2.74 | 15.4 | 4.97 | 0.44 | 1.6 | 1.22 | 6.91 | 0.053 | 31.28 |

The solution was made up by mixing 200 cc. of 0.1 M $H_3PO_4$ and 1,006.64 cc. of 0.02 M $Ca(OH)_2$. Samples were withdrawn daily. Solution was shaken once a day.

removed from time to time for analysis, the pH, and calcium and phosphorus contents being determined. The experimental technique was identical with that reported in the preceding paper. Calculations of the $PO_4^{\equiv}$ and $HPO_4^{=}$ ion concentrations were made as there described. For the first few days after mixing, samples were analyzed every 24 hours; after this the intervals were longer. The results are shown in Table I, and plotted in Figs. 1 and 2, where the values of the observed ion products (expressed as negative logarithms) are plotted against the time in days. The

value plotted for infinite time is the approximate equilibrium value obtained in another experiment where a similar solution was shaken continuously for 10 days. The horizontal lines

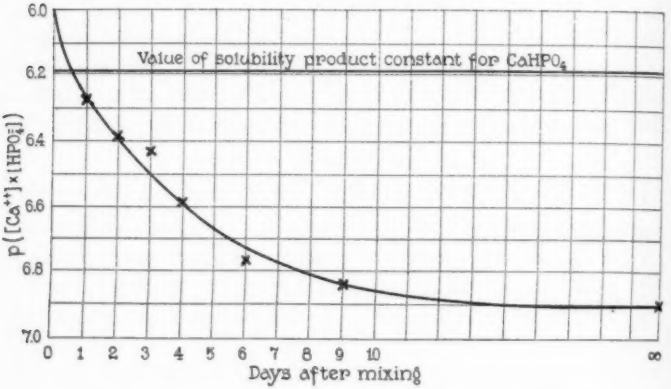


FIG. 1. Effect of time on the ion product $[Ca^{++}] \times [HPO_4^{=}]$.

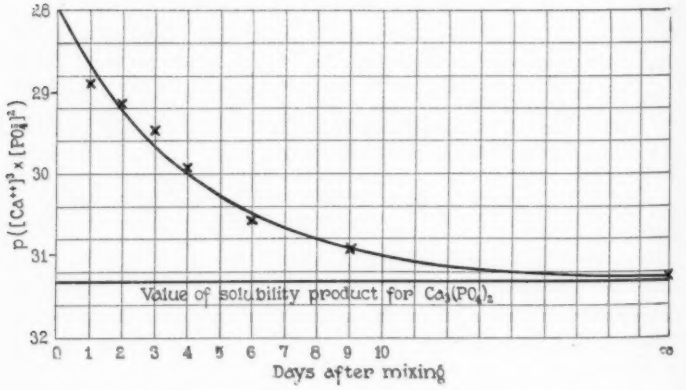


FIG. 2. Effect of time on the ion product $[Ca^{++}]^3 \times [PO_4^{=}]^2$.

representing the solubility product values are obtained from other experiments in which the stoichiometric solubility product was determined at approximately the same salt concentration.

It is quite clear from the above data that after the first 24 hours the ion product for $CaHPO_4$ is below the value of the solubility product constant for this salt. The solution is undersaturated, and as time goes on it becomes even more undersaturated. On the other hand, by extrapolating the curve to zero time, it is quite apparent that for, at least a short time after mixing, the ion product $CaHPO_4$ is above the value of the solubility product. Consequently one would expect precipitation of this salt to occur for a short time after mixing, but with further time the precipitate so formed should dissolve.

The ion product for $Ca_3(PO_4)_2$ on the other hand is always above the value of the solubility product constant, and it approaches the equilibrium value of this constant with considerable reluctance. The solution consequently remains supersaturated with respect to $Ca_3(PO_4)_2$ for many days.

The effect of the temporary precipitation of $CaHPO_4$ on the rate of precipitation of $Ca_3(PO_4)_2$ and on the hydrogen ion concentration of the solution may be more clearly visualized if we follow the equilibrium changes in the solution step by step. When the solutions are first mixed the solubility product constants of both $CaHPO_4$ and $Ca_3(PO_4)_2$ are exceeded; consequently both of the following reactions occur simultaneously:

$$(1) \qquad Ca^{++} + HPO_4^{=} \rightarrow CaHPO_4 \downarrow$$

$$(2) \qquad 3Ca^{++} + 2PO_4^{\equiv} \rightarrow Ca_3(PO_4)_2 \downarrow$$

There are reasons, however, why one would expect reaction (1) to proceed more rapidly than reaction (2). If we consider the reactions as written to represent the kinetic equations for the formation and precipitation of the solid salts, then reaction (1) is a second order reaction; its rate depends upon the simultaneous collision of only 2 ions. Reaction (2), however, may be considered to be a fifth order reaction;[2] its rate depends on the simultaneous collision of 5 ions, and the probability that 5 ions will collide simultaneously in a given period of time is very much smaller

[2] It is true that the existence of reactions of orders as high as the fifth have been questioned, and it is entirely possible that reaction (2) may prove to be composed of two or more consecutive reactions of lower order. These stages will, however, require time, and on this account one would still expect the velocity of the total reaction to be slower than that of reaction (1).

than the probability that 2 ions will so collide. Consequently it seems logical to suppose that reaction (1) outruns reaction (2) in velocity. Since, therefore, $HPO_4^=$ ions are being removed from the solution more rapidly than $PO_4^{\equiv}$ ions, it follows that the $H^+$ and $PO_4^{\equiv}$ ions will combine to form $HPO_4^=$ ions, in order to maintain the value of the ionization constant $K_3 = \frac{[H^+] \times [PO_4^{\equiv}]}{[HPO_4^=]}$, which reaction should reach equilibrium with great speed. Consequently we would expect to find the solution more alkaline than if precipitation of $CaHPO_4$ were not taking place. The conversion of $H^+$ and $PO_4^{\equiv}$ ions into $HPO_4^=$ ions to maintain the value of the ionization constant reduces the number of $PO_4^{\equiv}$ ions available for reaction (2), which is consequently slowed down still further. Before long, however, reaction (1) begins to reach completion. Its rate consequently slows down, and it is soon exceeded by reaction (2); $PO_4^{\equiv}$ ions are then being removed from the solutions more rapidly than $HPO_4^=$ ions, with the result that $HPO_4^=$ ions now must dissociate into $H^+$ and $PO_4^{\equiv}$ ions to maintain the value of the constant $K_3$.

The increase in acidity while this process is going on can be seen from the pH measurements in Table I. After a time so much $HPO_4^=$ is used up that the ion product $[Ca^{++}] \times [HPO_4^=]$ falls below the value of the solubility product constant for $CaHPO_4$. Solid $CaHPO_4$ then begins to dissolve, furnishing $Ca^{++}$ and $HPO_4^=$ ions, and thus assisting further the precipitation of $Ca_3(PO_4)_2$. Equilibrium cannot be reached until all the $CaHPO_4$ is dissolved.

From the foregoing explanation it seems clear that the rate of solution of the precipitated $CaHPO_4$ is a factor of great importance in delaying the final equilibrium. Most substances dissolve less rapidly than they precipitate, owing to the difficulties of maintaining free diffusion in the neighborhood of the solid particles. If Bassett's conception is correct that solid particles of $CaHPO_4$ become covered with a gelatinous coating of $Ca_3(PO_4)_2$, this would cause still further interference with diffusion, and would slow the rate of solution of $CaHPO_4$ even more. It seems, therefore, likely that the intermediate precipitation of $CaHPO_4$ is a factor of great importance in delaying equilibrium under these conditions.

On the other hand, it seems quite possible that this factor is not the only one responsible for the delay in equilibrium. A considerable part of this delay may be due to the inherent slowness of the reaction for the precipitation of tertiary phosphate, due perhaps to the fact that this is a fifth order reaction. The gradual increase in acidity could equally well be accounted for by a slow rate of precipitation alone. For, as $PO_4^{\equiv}$ ions are removed from solution, $HPO_4^{=}$ and $H_2PO_4^{-}$ ions must dissociate into $H^+$ and $PO_4^{\equiv}$ ions to maintain the value of the ionization constant. Side-tracking of some of the $Ca^{++}$ and $PO_4^{\equiv}$ ions in the form of secondary phosphate could easily explain the length of time required to reach equilibrium but *this alone would not explain the fact that ion products for tertiary phosphate,* $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, *greatly in excess of the value of the solubility product constant, can exist for considerable periods of time.*

This state of affairs can be more easily understood when we consider the factors which govern the rate of precipitation. The rate of any precipitation reaction is the resultant of the driving force of the reaction and of the forces which impede the progress of the reaction. The driving force depends upon the degree of supersaturation; in other words, on the ratio of the ion product to the solubility product constant, which may be shown by the equation:

$$\text{Driving force} = -\Delta F = RT \log \frac{\text{ion product}}{K_{s.p.}}$$

This expresses the amount of available work in calories which is liberated when the system passes from the supersaturated to the saturated state. Since this equation is a general one, it offers no explanation of the fact that the precipitation of $Ca_3(PO_4)_2$ occurs very much more slowly than that of other insoluble salts whose ion products exceed their solubility product constants by an equal amount. We must therefore fall back on the factors which impede precipitation as the cause of the behavior of $Ca_3(PO_4)_2$. There are many such possible factors. The effects of solid precipitate in the system and of mechanical agitation are, of course, well known. Marc (8) has found that certain foreign substances may catalyze such precipitation reactions, while other substances may act as poisons and greatly impede the process.

The order of the reaction is of great importance in this connection. As has been suggested above, it would seem most probable that the high order of the reaction is the chief factor in delaying the precipitation of $Ca_3(PO_4)_2$, for in the case of a salt composed of many ions, the probability of simultaneous collision of the appropriate ions in a given unit of time is very much smaller than for simpler salts.

When, however, constant conditions are maintained, and particularly as equilibrium is approached, the impeding effect of these various factors may be considered as constant, and the rate of precipitation may then be expressed by the equation:

$$\text{Rate of precipitation} = K_v \text{ [driving force]}$$

or

$$\frac{\text{d ion product}}{\text{d time}} = K_v\, RT \log \frac{\text{ion product}}{K_{s.p.}}$$

where $K_v$ is the velocity constant comprising the various factors impeding precipitation. From this equation one would expect that the rate of precipitation would steadily decrease in logarithmic fashion as the extent of supersaturation decreases, and that this rate should approach zero asymptotically as the value of the ion product approaches that of the solubility product constant, for when the ion product becomes identical with the $K_{s.p.}$, the $\log \frac{\text{ion product}}{K_{s.p.}} = \log 1 = 0$; consequently, there is no further driving force.

Inspection of the curve in Fig. 2 fulfills this expectation; that it is essentially logarithmic in character can easily be seen. We have not evaluated the constant $K_v$ from our data because the experimental conditions (agitation, etc.) are not comparable to those which occur in physiological systems.

We have then two factors which operate to cause a delayed equilibrium between $Ca_3(PO_4)_2$ and solutions from which it may be precipitated: the first, the intermediate precipitation of $CaHPO_4$, which later dissolves with great slowness; and the second, the inherently slow rate at which $Ca_3(PO_4)_2$ precipitates, due probably to the fact that this reaction is one of the fifth order.

Which of these two factors is of the greatest importance depends

TABLE II.

*Effect of Shaking Serum with $Ca_3(PO_4)_2$ at 38°C.*

| | pH | $Ca^{++}$ | $[Ca^{++}]$ | P | [P] | $[PO_4^{\equiv}]$ | $[PO_4^{\equiv}]^3$ | $[Ca^{++}]^3$ | $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ | $-\log$ product. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *mg. per 100 cc.* | *mols* | *mg. per 100 cc.* | *mols* | *mols* | | | | |
| **I. Human. T. C. G.** | | | | | | | | | | |
| Before shaking. | 7.40 | 10.3 | $2.58 \times 10^{-3}$ | 3.2 | $1.03 \times 10^{-3}$ | $7.44 \times 10^{-9}$ | $5.54 \times 10^{-17}$ | $1.71 \times 10^{-8}$ | $9.47 \times 10^{-25}$ | 24.02 |
| After shaking 1 wk. with $Ca_3(PO_4)_2$. | 7.70 | 1.8 | $4.5 \times 10^{-4}$ | 1.5 | $4.84 \times 10^{-4}$ | $8.08 \times 10^{-9}$ | $6.53 \times 10^{-17}$ | $9.1 \times 10^{-11}$ | $5.94 \times 10^{-27}$ | 26.23 |
| **II. Human. C.O' D.** | | | | | | | | | | |
| Before shaking. | 7.40 | 10.2 | $2.55 \times 10^{-3}$ | 3.5 | $1.13 \times 10^{-3}$ | $8.17 \times 10^{-9}$ | $6.6 \times 10^{-17}$ | $1.66 \times 10^{-8}$ | $1.11 \times 10^{-24}$ | 23.95 |
| After shaking 1 wk. with $Ca_3(PO_4)_2$. | 7.70 | 2.5 | $6.25 \times 10^{-4}$ | 1.45 | $4.68 \times 10^{-4}$ | $7.82 \times 10^{-9}$ | $6.11 \times 10^{-17}$ | $2.44 \times 10^{-10}$ | $1.49 \times 10^{-26}$ | 25.83 |
| **III. Horse. 5515-H.** | | | | | | | | | | |
| Before shaking. | 7.35 | 11.0 | $2.75 \times 10^{-3}$ | 3.4 | $1.10 \times 10^{-3}$ | $6.79 \times 10^{-9}$ | $4.61 \times 10^{-17}$ | $2.08 \times 10^{-8}$ | $9.59 \times 10^{-25}$ | 24.02 |
| After shaking 2 wks. with $Ca_3(PO_4)_2$. | 7.40 | 2.7 | $6.75 \times 10^{-4}$ | 2.1 | $6.77 \times 10^{-4}$ | $4.90 \times 10^{-9}$ | $2.40 \times 10^{-17}$ | $3.08 \times 10^{-10}$ | $7.38 \times 10^{-27}$ | 26.13 |

upon the existing conditions. The second factor would seem to be directly concerned under all conditions. The first factor, however, would appear to become of great importance only when the conditions are such that the solubility product for $CaHPO_4$ is exceeded, and the importance which this factor assumes would depend on the extent to which the solubility product for $CaHPO_4$ is exceeded in the early stages of the precipitation.

The fact that supersaturation with $Ca_3(PO_4)_2$ occurs in blood serum as well as in inorganic solutions is clearly demonstrated by the solubility experiments, partly described in the preceding paper. *When fresh sterile blood serum is shaken at 38°C. in the absence of solid $Ca_3(PO_4)_2$ little or no reduction in the ion product occurs, but when the shaking is carried out in contact with this salt, a marked reduction occurs in the course of time, quite analogous to that found with inorganic solutions.* This is clearly shown in Table II, in which analyses of serum before and after such shaking are given.

That blood serum is normally greatly[3] supersaturated with $Ca_3(PO_4)_2$ seems unquestionable, and it is highly probable that the other body fluids, including urine, are similarly supersaturated with this salt. This state of supersaturation should be clearly differentiated from the increase in solubility due to inorganic salts and proteins, which was taken up in Paper I. Salts and proteins increase the equilibrium value of the stoichiometric $K_{s.p.}$, *but in the present case we have concentrations in excess of the equilibrium value, and consequently a true state of supersaturation.* The question remains as to the possible significance of this state of supersaturation. Quite obviously the body fluids thus constitute a reservoir for calcium and phosphorus, which, however, becomes available for the production of solid $Ca_3(PO_4)_2$ only (*a*) in tissues where some agent is present which accelerates precipitation, or (*b*) in such fluids as are actually in contact with solid $Ca_3(PO_4)_2$. It is obvious that this latter condition is fulfilled in the tissue fluids bathing the bone matrix.

The ability of the blood to remain supersaturated with $Ca_3(PO_4)_2$ would seem to be of great importance in maintaining the normal nervous irritability of the organism. Thus Howland and Marriott (9) and others have shown that symptoms of active tetany

[3] More than 200 per cent in terms of the molar solubility of $Ca_3(PO_4)_2$.

appear regularly when the serum calcium falls below 7 mg. per 100 cc. From the data in Table II it can be seen that shaking with $Ca_3(PO_4)_2$ reduces the serum calcium from its normal value of 10 to 11 mg. per 100 cc. not only to the tetany level of 7, but even to 2 or 1.5 mg. per 100 cc. Were the blood unable to hold such excessive amounts of calcium in solution, the entire human race, not to mention the lower animals, would be in a continuous state of tetany.

It may now be asked, how does it happen that such large quantities of calcium phosphate get into solution. *In vitro* this state of affairs is produced by mixing more concentrated solutions, and it would seem that a somewhat similar process might occur in the body. The various foods containing calcium salts and inorganic phosphates are thoroughly mixed in the stomach, where the reaction is so acid that considerable quantities of these substances are held in solution. The reaction of the intestinal contents is somewhat more alkaline, but there is reason to believe (10) that in the small intestine, at least, it remains on the acid side of neutrality. During absorption from the intestine, a sudden shift in reaction to the alkaline side occurs as the products absorbed are brought to the pH of the blood (7.4). A comparatively large quantity of $PO_4^{\equiv}$ ion is thus suddenly produced from $H_2PO_4^-$ and $HPO_4^=$ ions. This change in reaction, which is probably controlled by other factors than phosphates, occurs too rapidly for the precipitation equilibria between $Ca^{++}$ and $PO_4^{\equiv}$ ions to keep pace. The product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ rises so rapidly that precipitation of $Ca_3(PO_4)_2$ lags far behind, and thus a supersaturated solution is formed.

## SUMMARY.

1. It is shown that the precipitation of tertiary calcium phosphate occurs so slowly that solutions of this salt may remain supersaturated for many days.

2. A quantitative study of the ion products for $CaHPO_4$ and $Ca_3(PO_4)_2$ with the progress of time shows that the delay in final equilibrium is produced by two factors: (1) The transitory precipitation of secondary calcium phosphate; and (2) the inherent slowness of the precipitation of $Ca_3(PO_4)_2$, which is perhaps due to the fact that this is a reaction of the fifth order.

3. It is shown that blood serum is normally supersaturated with tertiary calcium phosphate to the extent of more than 200 per cent. The biological significance of this fact is discussed.

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# STUDIES IN CALCIFICATION.

## III. A QUANTITATIVE STUDY OF THE EQUILIBRIA CONCERNED WITH THE CALCIFICATION OF BONE.

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In discussing the problem of bone calcification one must bear in mind the anatomical relations between the bone matrix, the osteoblasts, and the blood capillaries. The osteoblasts may be considered as resting in tissue spaces which are in contact with the amorphous bone matrix on the one hand, and with the walls of the blood vessels on the other. The bone matrix consists of a protein substance, ossein or collagen, which constitutes about one-third of its mass. The remaining two-thirds consist of inorganic matter: calcium, phosphorus, carbonate, and magnesium, with perhaps small traces of alkali salts. Only the calcium and phosphorus are present in relatively large amounts, and the ratio in which these elements exist is almost exactly that required to form tertiary calcium phosphate $Ca_3(PO_4)_2$. Thus it is usually stated that about 85 per cent of the inorganic matter of bone consists of this salt, about 14 per cent is calcium carbonate, and the remainder magnesium carbonate and various alkali salts.

There have been two chief theories about the production of the calcified bony matrix. Some authors claim that both the collagen and the lime salts are secreted by the osteoblasts. Others, however, while admitting the likelihood that the collagen is produced by the osteoblasts, believe that the lime salts are laid down in the matrix—either separately or as a double salt—by a simple process of precipitation. In either case it is quite obvious that the inorganic salts must ultimately be derived from the blood stream, and it is consequently not surprising to find, as Howland and Kramer (1) have demonstrated in infants, that

when either the calcium or the phosphorus or both of these elements are sufficiently diminished in the blood serum, a condition of impaired calcification—active rickets—is always present.

It is not our purpose to discuss the evidence for or against these two theories; however, it may perhaps be worth while to point out that, in the complete absence of any data on the solubility of $CaCO_3$ and $Ca_3(PO_4)_2$ in biological fluids, to postulate a process of precipitation is at best but a shrewd surmise. The ultimate proof of such a theory must rest until it can be shown that calcification of bone proceeds only when the ion products of the two insoluble salts in the tissue fluid exceed the solubility product constant for these salts in that particular medium.

At present it seems quite impractical to investigate the composition of the fluid in contact with bone matrix. We have therefore to fall back on the blood serum as offering the nearest approach to this. To correlate the solubility data and ion products in serum with the process of calcification would at least offer suggestive, if not conclusive, evidence for the deposition of these bone salts by precipitation.

The present study was confined to that salt which constitutes the major part of the inorganic matter of bone—tertiary calcium phosphate. The product of the ions which constitute this salt, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, was calculated from data of Howland and Kramer (1, 2) on the blood serum of infants and rats with active and healing rickets. Some normal cases have been included for the sake of comparison. The object in view was to ascertain the relation between the ion products and the solubility product constant for $Ca_3(PO_4)_2$ in blood serum when calcification was proceeding normally or when it was impaired.

The ion products were calculated as in Paper I. The calcium was assumed to be completely dissociated. $[PO_4^{\equiv}]$ was calculated from the equation derived in Paper I:

$$[PO_4^{\equiv}] = \frac{[P]\ K_1\ K_2\ K_3}{[H^+]^3 + [H^+]^2K_1 + [H^+]K_1K_2 + K_1K_2K_3}$$

Where $[P]$ = the molar concentration of total phosphorus.
$K_1$, $K_2$, and $K_3$ = the first, second, and third dissociation constants of $H_3PO_4$.

$[H^+]$ = hydrogen ion concentration. This was assumed to be pH = 7.35 in all cases, as it has never been shown to deviate from the normal in rickets.

The ion products thus calculated are stoichiometric ion products, and are, therefore, entirely comparable to the stoichio-

TABLE I.

*Ion Products in Active and Healing Rickets and Normal Infants.*

| Ca | $[Ca^{++}]$ | P | [P] | Ca × P | $[PO_4^{\equiv}]$ | $[PO_4^{\equiv}]^3 \times 10^{17}$ | $[Ca^{++}]^3 \times 10^9$ | $[Ca^{++}]^3 \times [PO_4^{\equiv}]^3 \times 10^{26}$ | − log product. | Condition. |
|---|---|---|---|---|---|---|---|---|---|---|
| *mg. per 100 cc.* | M × $10^3$ | *mg. per 100 cc.* | M × $10^3$ | *mg. per 100 cc.* | M × $10^3$ | | | | | |
| 11.0 | 2.75 | 6.8 | 2.19 | 74.8 | 13.6 | 18.4 | 2.08 | 38.3 | 23.42 | Healing rickets. |
| 10.3 | 2.58 | 6.0 | 1.94 | 61.8 | 12.0 | 14.4 | 1.71 | 24.5 | 23.61 | " " |
| 10.2 | 2.55 | 6.0 | 1.94 | 61.2 | 12.0 | 14.4 | 1.66 | 23.8 | 23.62 | " " |
| 10.0 | 2.50 | 6.0 | 1.94 | 60.0 | 12.0 | 14.4 | 1.56 | 22.3 | 23.65 | " " |
| 9.8 | 2.45 | 6.1 | 1.97 | 59.8 | 12.2 | 14.9 | 1.47 | 21.9 | 23.66 | " " |
| 9.7 | 2.43 | 6.2 | 2.00 | 60.1 | 12.4 | 15.3 | 1.42 | 21.8 | 23.66 | " " |
| 9.8 | 2.45 | 5.3 | 1.71 | 51.9 | 10.6 | 11.2 | 1.47 | 17.9 | 23.75 | " " |
| 10.2 | 2.55 | 4.5 | 1.45 | 45.9 | 8.98 | 8.06 | 1.66 | 13.4 | 23.87 | Normal. |
| 8.7 | 2.18 | 4.7 | 1.52 | 40.9 | 9.38 | 8.81 | 1.03 | 9.07 | 24.04 | Healing rickets. |
| 8.0 | 2.00 | 5.2 | 1.68 | 41.6 | 10.4 | 10.6 | 0.80 | 8.64 | 24.06 | Normal. |
| 9.5 | 2.38 | 3.8 | 1.23 | 36.1 | 7.59 | 5.76 | 1.34 | 7.72 | 24.11 | Active rickets. |
| 7.2 | 1.80 | 5.3 | 1.71 | 38.2 | 10.6 | 11.2 | 0.58 | 6.53 | 24.19 | " " |
| 9.7 | 2.43 | 3.0 | 0.97 | 29.1 | 5.99 | 3.59 | 1.42 | 5.10 | 24.29 | " " |
| 6.2 | 1.55 | 5.5 | 1.77 | 34.1 | 11.0 | 12.1 | 0.37 | 4.49 | 24.35 | " " |
| 9.7 | 2.43 | 2.6 | 0.84 | 25.2 | 5.19 | 2.70 | 1.42 | 3.83 | 24.42 | " " |
| 5.8 | 1.45 | 5.0 | 1.61 | 29.0 | 9.98 | 9.97 | 0.31 | 3.04 | 24.53 | " " |
| 10.5 | 2.63 | 1.8 | 0.58 | 18.9 | 3.60 | 1.29 | 1.81 | 2.34 | 24.63 | " " |
| 4.9 | 1.23 | 5.0 | 1.61 | 24.5 | 9.98 | 9.97 | 0.18 | 1.83 | 24.74 | " " |
| 9.8 | 2.45 | 1.6 | 0.52 | 15.7 | 3.19 | 1.02 | 1.47 | 1.50 | 24.82 | " " |
| 9.6 | 2.40 | 0.8 | 0.26 | 7.7 | 1.60 | 0.255 | 1.38 | 0.35 | 25.45 | " " |

metric solubility product constant evaulated in Paper I. Results for children and for rats are given in Tables I and II.[1]

[1] A few of these cases have been taken from unpublished data of Howland and Kramer.

*Comment.*

Tables I and II show clearly that the ion product in healing rickets, or in the absence of rickets, is always definitely larger than when the disease is active. When the disease is healing or

TABLE II.

*Ion Products in Active and Healing Rickets and Normal Rats.*

| Rat No. | Ca | $[Ca^{++}]$ | P | $[P]$ | Ca × P | $[PO_4^{\equiv}]$ | $[PO_4^{\equiv}]^3 \times 10^{17}$ | $[Ca^{++}]^3 \times 10^6$ | $[Ca^{++}]^3 \times [PO_4^{\equiv}]^3 \times 10^{23}$ | − log product (p product). | Condition. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *mg. per 100 cc.* | M ×10³ | *mg. per 100 cc.* | M ×10³ | *mg. per 100 cc.* | M ×10³ | | | | | |
| 1495–1498 | 10.0 | 2.50 | 8.8 | 2.84 | 88.0 | 17.6 | 30.9 | 1.56 | 48.2 | 23.32 | Normal. |
| 1409–1413 | 9.8 | 2.45 | 8.0 | 2.58 | 78.4 | 16.0 | 25.5 | 1.47 | 37.5 | 23.43 | " |
| 1516–1519 | 9.1 | 2.28 | 8.2 | 2.65 | 74.6 | 16.4 | 26.8 | 1.18 | 31.6 | 23.50 | " |
| 1557–1562 | 10.3 | 2.58 | 5.7 | 1.84 | 58.7 | 11.4 | 13.0 | 1.71 | 22.2 | 23.65 | Healing rickets. |
| 1721–1724 | 8.2 | 2.05 | 7.0 | 2.26 | 57.4 | 14.0 | 19.6 | 0.86 | 16.9 | 23.77 | " " |
| 1586–1588 | 10.4 | 2.60 | 4.4 | 1.42 | 45.7 | 8.80 | 7.73 | 1.76 | 13.6 | 23.87 | " " |
| 1501–1504 | 10.2 | 2.55 | 4.4 | 1.42 | 44.9 | 8.80 | 7.73 | 1.66 | 12.8 | 23.89 | " " |
| 1589–1593 | 6.8 | 1.70 | 8.0 | 2.58 | 54.4 | 16.0 | 25.5 | 0.49 | 12.5 | 23.90 | Normal. |
| 1663–1667 | 10.4 | 2.60 | 4.1 | 1.32 | 42.6 | 8.19 | 6.71 | 1.76 | 11.8 | 23.93 | Healing rickets. |
| 1558–1561 | 6.5 | 1.63 | 8.0 | 2.58 | 52.0 | 16.0 | 25.5 | 0.43 | 10.9 | 23.96 | " " |
| 1547–1552 | 10.3 | 2.58 | 3.5 | 1.13 | 36.1 | 6.99 | 4.89 | 1.71 | 8.36 | 24.08 | " " |
| 1725–1726 | 5.5 | 1.38 | 8.0 | 2.58 | 44.0 | 16.0 | 25.5 | 0.26 | 6.63 | 24.18 | " " |
| 1686–1689 | 9.6 | 2.40 | 3.2 | 1.03 | 30.7 | 6.39 | 4.08 | 1.38 | 5.63 | 24.25 | Active rickets. |
| 1735–1739 | 10.1 | 2.53 | 2.7 | 0.87 | 27.3 | 5.39 | 2.91 | 1.61 | 4.69 | 24.33 | " " |
| 1376–1378 | 10.5 | 2.63 | 2.4 | 0.77 | 25.2 | 4.79 | 2.30 | 1.81 | 4.15 | 24.38 | " " |
| 1842–1844 | 4.4 | 1.10 | 8.0 | 2.58 | 35.2 | 16.0 | 25.5 | 0.13 | 3.39 | 24.47 | " " |
| 1855–1858 | 4.1 | 1.03 | 8.6 | 2.77 | 35.3 | 17.2 | 29.5 | 0.11 | 3.19 | 24.50 | " " |
| 1845–1846 | 4.5 | 1.13 | 7.2 | 2.32 | 32.4 | 14.4 | 20.8 | 0.14 | 2.96 | 24.53 | " " |
| 1781–1784 | 10.0 | 2.50 | 2.0 | 0.65 | 20.0 | 3.99 | 1.60 | 1.56 | 2.49 | 24.60 | " " |
| 1776–1780 | 10.0 | 2.50 | 1.8 | 0.58 | 18.0 | 3.60 | 1.29 | 1.56 | 2.02 | 24.70 | " " |

absent the ion product is usually greater than $8.0 \times 10^{-25}$ (p product less than 24.10), while in active rickets the product is usually below this figure (p product greater than 24.10). The dividing line is not, however, an absolutely sharp one.

When, however, one compares these products with the solubility product constant for $Ca_3(PO_4)_2$ in blood serum, which was found to be about $1 \times 10^{-26}$ ($pK_{s.p.} = 26.0$), it is quite clear that even in active rickets the product is considerably greater than that required to precipitate tertiary calcium phosphate. At first glance it might seem surprising that ion products in excess of the solubility product constant are found associated with a condition in which the deposition of $Ca_3(PO_4)_2$ is so clearly at fault. It was, however, pointed out in the preceding paper of this series that the precipitation of tertiary calcium phosphate is a comparatively slow process. Although this salt is precipitated with moderate rapidity when the ion product is *greatly* in excess of the solubility product constant, precipitation proceeds with great slowness when the ion product is only slightly in excess of the value of the constant. The rate of precipitation depends on how much the value of the solubility product constant is exceeded.

Thus it would seem quite possible that even in active rickets calcification is proceeding, but at a rate so slow that it is not ordinarily detected. Certain observations recently made by Shipley[2] on the bones of rats which have been kept for a long time on McCollum's (3) Diet 3143 offer very suggestive evidence of a slow gradual deposition of lime salts, even during active rickets. These bones have a broad rachitic metaphysis. During the first 6 weeks of the condition, which may be regarded as the acute stage of the disease, this part of the bone is free from calcium. As the condition becomes chronic, however, the metaphysis not infrequently becomes peppered throughout with fine deposits of lime salts.

Thus there is histological as well as chemical evidence for believing that even in active rickets calcification is proceeding, but at an extraordinarily slow rate. When the deposition of $Ca_3(PO_4)_2$ is retarded beyond a certain point, the growth of new bone exceeds it in rapidity, and an inadequately calcified tissue, osteoid tissue, is formed which has been taken as pathognomonic of rickets.

We have then a new conception of the immediate cause of the

[2] Shipley, P. G., personal communication.

bone changes in rickets, and we can define rickets: not as a state in which the concentrations of calcium and phosphate are so low that $Ca_3(PO_4)_2$ cannot be precipitated, but as a state in which, as a result of lowered ion concentrations, $Ca_3(PO_4)_2$ is deposited so slowly that new bone production exceeds it in rapidity, and consequently uncalcified bone or osteoid tissue is produced.

One can thus readily understand the fact that the serum of normal adults contains concentrations of calcium and phosphate similar to those found in active rickets in infants. In adults the growth of bone is either at a standstill or is exceedingly slow; consequently an exceedingly slow rate of deposition of $Ca_3(PO_4)_2$ is required to keep pace with it, and no pathological changes occur. It is only under special conditions of strain, such as occur in uniting fractures when rapid calcification is demanded, that the individuals with the lowest concentration experience difficulty (4).

In the light of these observations it may be well to consider the significance in rickets of the product of the calcium $\times$ total phosphorus concentrations when expressed in mg. per 100 cc. This product was first suggested by Howland and Kramer (1) in 1922 as an empirical criterion of the activity of a rachitic process, and it has been widely and quite justifiably used as such during the past few years. When, however, one compares this product with the stoichiometric ion product $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ in Tables I and II, it is apparent that these two functions are not parallel. In general it holds true that a high ion product is associated with a high empirical Ca $\times$ P product and *vice versa*, and hence the empirical product remains a rough guide to the true ion concentration and to the activity of the rachitic process. It is, however, a less accurate guide than the stoichiometric ion product for several reasons, the chief of which is that the empirical product does not give enough weight to the calcium concentration. Thus in Table II it will be noticed in those cases of active rickets associated with low calcium that the empirical product Ca $\times$ P is only slightly reduced, and remains between 30 and 40, the range which has been considered an intermediate range, sometimes associated with active and sometimes with healing rickets. In all these cases, however, the ion product is reduced to a much

greater extent, well beyond the doubtful range, which is in harmony with the observation that rats with such concentrations exhibit signs of active rickets with great regularity. Table I shows this same variation in a somewhat less striking manner.

The stoichiometric ion product has the additional advantage that physiological and pathological changes in pH are entirely accounted for. As can be seen from Table III comparatively small variations of pH produce considerable change in that fraction of the total phosphorus ionized as $PO_4^{\equiv}$.

The chief objection to the use of the ion product as a criterion of the activity of rickets is the inconvenience of making the

TABLE III.

| pH | $[PO_4^{\equiv}]$ when [P] = 1. | $p[PO_4^{\equiv}]$ when [P] = 1. |
|---|---|---|
| 7.10 | $2.80 \times 10^{-6}$ | 5.55 |
| 7.15 | $3.31 \times 10^{-6}$ | 5.48 |
| 7.20 | $3.89 \times 10^{-6}$ | 5.41 |
| 7.25 | $4.55 \times 10^{-6}$ | 5.34 |
| 7.30 | $5.32 \times 10^{-6}$ | 5.27 |
| 7.35 | $6.19 \times 10^{-6}$ | 5.21 |
| 7.40 | $7.19 \times 10^{-6}$ | 5.14 |
| 7.45 | $8.32 \times 10^{-6}$ | 5.08 |
| 7.50 | $9.61 \times 10^{-6}$ | 5.02 |
| 7.55 | $1.11 \times 10^{-5}$ | 4.96 |
| 7.60 | $1.27 \times 10^{-5}$ | 4.90 |
| 7.65 | $1.46 \times 10^{-5}$ | 4.84 |
| 7.70 | $1.67 \times 10^{-5}$ | 4.78 |

calculation. The difficulty is largely due to the habit of recording concentrations of calcium and phosphorus in mg. per 100 cc., units without any physicochemical significance. Were such concentrations recorded in terms of mols or, better still (following the custom so well established for hydrogen ions), as negative logarithms of molar concentrations: $p[Ca^{++}]$ and p[P], such calculations would then become exceedingly simple. $p[PO_4^{\equiv}]$ which can be read from the third column of Table III could easily be obtained from p[P] by the addition of a constant. Then by adding $3p[Ca]$ and $2p[PO_4^{\equiv}]$ the negative logarithm of the ion

product, p product, is directly obtained. A sample calculation is given below:

$Ca = 2.5 \times 10^{-3}$ molar (= 10 mg. per 100 cc.)
$\log [Ca] = \bar{3}.40$
$p [Ca] = 2.60$
$P = 1.3 \times 10^{-3}$ molar (= 4 mg. per 100 cc.)
$\log [P] = \bar{3}.11$
$p [P] = 2.89$
$p[PO_4^{\equiv}]$, at pH 7.35, $= 2.89 + 5.21 = 8.10$
p Product $= (3 \times 2.60) + (2 \times 8.10) = 24.00$

From the data which we have presented it seems clear that the degree of supersaturation is a factor of the greatest importance in governing the deposition of $Ca_3(PO_4)_2$ from fluids containing this salt in solution. The reduced degree of supersaturation of the blood serum in active rickets is found so consistently and is of such a magnitude that it is difficult to escape the belief that this is the immediate factor which hinders the deposition of $Ca_3(PO_4)_2$ in the bones in rickets. On the other hand, it should be emphasized that this is not necessarily the *only* factor influencing the deposition of this salt. In the preceding paper we have referred to the work of Marc (5) who has shown that a great variety of substances may affect the rate of deposition from supersaturated solutions. Some substances act as catalysts and increase the rate of deposition, while others may act as poisons and delay the process very greatly. It seems quite possible that some such phenomena may explain why $Ca_3(PO_4)_2$ is so readily deposited in certain tissues, and not at all in others.

SUMMARY.

1. The ion product for tertiary calcium phosphate, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, in the blood serum is smaller in active rickets than when the disease is absent or healing.

2. Even in active rickets this ion product is greater than that required to precipitate $Ca_3(PO_4)_2$.

3. It is suggested that calcification is not completely arrested in rickets, but that it is so retarded that bone growth exceeds it in rapidity. Thus the inadequately calcified osteoid tissue, so characteristic of rickets, is produced.

4. The ion product, $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$, is a more accurate criterion of the rate of deposition of $Ca_3(PO_4)_2$ and hence of the activity of rickets than is the empirical Ca × P product.

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# THE JENDRASSIK REACTION FOR WATER-SOLUBLE B.

## CONCERNING VICTOR E. LEVINE'S CRITICAL STUDY.

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Levine (1924) claimed in his conclusions that the ferric ferricyanide reaction proposed by Jendrassik as a specific color test for water-soluble B "is given by phenols and may serve as a test for phenols in the absence of a few positively reacting non-phenolic compounds," and that "the finding that active preparations of water-soluble B do not give a positive Millon or Liebermann reaction is no proof of the fact that vitamin B is not a phenol, since neither the Millon nor the Liebermann reaction is given by all phenols."

In a study of the significance of the color test for water-soluble B proposed by Jendrassik, published in January, 1924 (Bezssonoff, 1924), and not mentioned by Levine, I have already claimed, that the ferric ferricyanide reaction used by Jendrassik is a test for very different phenols: monophenol, hydroquinone, catechol, phloroglucin, and pyrogallol. I have specially examined the value of Millon and Libermann reactions for phenols and, like Levine, concluded that Jendrassik's finding that an active preparation of water-soluble B does not give a positive Millon or Liebermann reaction is no proof of the fact that vitamin B is not a phenol.

But there is one *essential* difference between Levine's observation and mine. Studying the characteristic value of the ferric ferricyanide reaction, I have not only used the observation time (10 minutes) indicated by Jendrassik but also, in conformity with Brouardel and Boutmy (1881) and Tanret (1881), seconds (10 seconds). In this way it was possible to note the existence of a great difference of sensitiveness of the ferric ferricyanide



XUM

reaction towards the mono- and polyphenols; and specially ortho- and para-polyphenols.

This reaction is less sensitive for the monophenols: the phenol concentration required is 5 per cent; for phloroglucin, 1/1,000; for ortho- and para-diphenols and pyrogallic acid, 1/200,000.

It is easy to see that in dealing with typical reactions for a vitamin, *only substances which possess a very great sensitiveness for this reaction are of interest.*

## CONCLUSIONS.

1. The essential part of the first claim of Levine (November, 1924): "the ferric ferricyanide reaction proposed by Jendrassik is not a specific test for water-soluble B. It is given by phenol. . . ." was contained in Bezssonoff's study (January, 1924).

2. The main difference between the observations of Levine and Bezssonoff consists in Bezssonoff's indication on the special and great sensitiveness of the ferric ferricyanide reaction for the ortho- and para-polyphenols.

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# THE JENDRASSIK REACTION FOR VITAMIN B WITH REFERENCE TO THE WORK OF BEZSSONOFF AND OF LEVINE.

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In January, 1924, there appeared an article by Nikolaï Bezssonoff relative to the Jendrassik reaction for vitamin B.[1] The November, 1924, issue of this Journal contained an article[2] dealing also with the Jendrassik reaction and submitted by us for publication July 17, 1924.

We are sorry to have overlooked Bezssonoff's paper. His paper and ours differ, however, in content and in conclusions reached. The only point common to both contributions concerns the conclusion relative to the fact that the inability of active vitamin B preparations to give a positive Millon or Liebermann reaction is no proof that vitamin B is not a phenol.

Bezssonoff asserts his priority to the claim that the ferric ferricyanide reaction proposed by Jendrassik[3] for vitamin B is a general test for phenols. We fail to discover any statement or any experimental proof in his original paper to substantiate this claim. He has investigated only four phenols. We, on the other hand, have tested 57 phenols, selected to represent all types—monophenols as well as monophenols carrying halogens or nitro, amino, alcohol, aldehyde, or carboxyl groups, monophenolic ethers, diphenols and their derivatives, triphenols, phenolic glucosides, and phenolic alkaloids. We wish to state emphatically that we do not claim, as Bezssonoff asserts, that the

[1] Bezssonoff, N., *Bull. Soc. chim. biol.*, 1924, vi, 35.
[2] Levine, V. E., *J. Biol. Chem.*, 1924-25, lxii, 157.
[3] Jendrassik, A., *J. Biol. Chem.*, 1923, lvii, 129.

Jendrassik reaction is a general test for phenols. We mention the fact[4] that salicylic acid, acetyl salicylic acid, anisole, anisic acid, heliotropine, heroin, *m*-dinitrophenol, dinitroaminophenol, and nitroso-β-naphthol are negative. To quote from our paper:[5] "At first we felt that the test would be specific for phenols, but further work showed us that it proved also positive with certain non-phenolic compounds, such as thiourea, uric acid, paraformaldehyde, *p*-dimethyl aminobenzaldehyde, Michler's ketone, sodium taurocholate, hydroxylamine hydrochloride, benzidine, toluidine, *p*-phenylenediamine hydrochloride, α- and β-naphthylamine."

According to Bezssonoff, the chief claim of Levine is that the Jendrassik reaction serves as a test for phenols. This is not the chief claim. The reactivity of the reagent for phenols and other organic compounds is merely incidental to the fact that the Jendrassik reaction is not applicable as a test for water-soluble B. The findings that the Jendrassik reaction is non-specific is neither to be credited to Bezssonoff nor to us. Jendrassik himself recognized the non-specificity of his reaction. To quote from his paper: "Brouardel and Boutmy described in 1881 the formation of a blue color by the reduction of ferric ferricyanide as a specific reagent for ptomaines in contrast with alkaloids. Tanret and Beckurts have shown that some of the alkaloids and glycosides are able to bring about the same reaction." Nevertheless, Jendrassik believed in the specificity of the reagent as applied to biologic materials, for he claimed that sugars, amino acids, and phenols—compounds ordinarily found in the biologic organism—do not give the reaction. We find that not only sugars but products likely to be obtained from their fragmentation—aldehydes, ketones, and organic acids—do not react. In addition, we emphasize the wide range of the Jendrassik reagent and show that certain compounds of biologic occurrence —phenol, ortho- and para-cresol, tyrosine, pyrocatechol, adrenalin, hydroquinone, uric acid, gallic acid, tannic acid, various glucosides, and alkaloids—give a positive reaction. The presence of these biologic compounds render the Jendrassik reaction inapplicable as a test for vitamin B. Incidently we may men-

[4] Levine,[2] p. 158.
[5] Levine,[2] p. 159.

tion that, contrary to our findings, uric acid is reported by Bezssonoff to react negatively.

Bezssonoff points out one essential difference between his observations and ours. He concludes that ortho-parapolyphenols show a greater sensitiveness to the ferric ferricyanide reaction. This general conclusion with reference to relative sensitivity is based, however, upon tests with one monophenol, one orthodiphenol, and one para-diphenol.

Bezssonoff has mentioned one point of difference in the two papers. He has overlooked another. We refer to the effect of alkali. The destructibility by alkali, which is a characteristic of vitamin B, phenols, and non-phenols, such as uric acid, thiourea, etc., is perhaps the most important finding recorded in our paper. We treated with alkali 57 compounds—45 phenols and 12 non-phenols—that gave positive reactions with the Jendrassik procedure. After neutralization and acidification with acetic acid the Jendrassik reaction became negative. Phenols, responding to the Millon and to the Liebermann reaction, also failed to do so after alkali treatment. As stated in our final conclusions, alkali reacts with phenols to give derivatives or fragments that no longer respond to the ordinary phenol tests. Phenols resemble vitamin B with respect to the destructive effect of alkali. The use of alkali cannot therefore serve to differentiate vitamin B from all other compounds that give a positive Jendrassik reaction.

XUM

# ON THE PROBABLE DIMENSIONS OF THE MOLECULE AND MOLECULAR WEIGHT OF CRYSTALLINE EGG ALBUMIN.

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PLATE 1.

(Received for publication, May 7, 1925.)

In the last few years, a method has been devised by which the dimensions of molecules of certain colloidal substances could be measured.[1,2] This method was applied, with the use of sodium oleate, the molecular weight of which is well known, to the determination of the constant $N$ of Avogadro, and yielded a value very close to the theoretical one ($6.004 \times 10^{23}$) and differing by less than 1.0 per cent from that, universally accepted as the best, of Millikan[3] ($6.062 \times 10^{23}$). The dimensions of the sodium oleate molecules were found to be $12.30 \times 7.56 \times 6.64$ Ångström units ($10^{-8}$ cm.). It is clear that should this method prove to be applicable to other substances, such as proteins, for example, it would make it possible to calculate the molecular weight, the constant $N$ being known. The purpose of this paper is to report the results of the application of this method to crystalline egg albumin.

## EXPERIMENTAL.

The principle of the method is to study the static surface tension of dilute solutions of the substances at concentrations from 1/10,000 to 1/1,000,000, and to detect the minima of the surface tension which have been shown to correspond to organized molecular layers, mainly monolayers. For this purpose the du

[1] du Noüy, P. L., *Phil. Mag.*, 1924, xlviii, 264; *J. Exp. Med.*, 1922, xxxv, 575, 707; xxxvi, 115, 547; 1923, xxxvii, 659; xxxviii, 87; 1924, xxxix, 37, 717; xl, 129, 133; xli, 663.

[2] du Noüy, P. L., *J. Gen. Physiol.*, 1923–24, vi, 625.

[3] du Noüy, P. L., *Phil. Mag.*, 1924, xlviii, 670; *J. phys.* (in press).



XUM

Noüy tensiometer is used, in connection with specially built tables,[2] on which the solutions are kept perfectly immobile (Fig. 1). Forty-four watch-glasses are placed in a row on each table, and the tensiometer is rolled on its rails, in front of each of them successively. Thus a very large number of measurements can be made rapidly. All watch-glasses are chosen so as to have very nearly the same radius of curvature, and exactly 2 cc. of liquid are poured onto each of them. 2 hours are allowed to elapse before the measurements are made, so as to give time for adsorption to take place.

It is hardly necessary to add that the greatest care was taken in the preparation of the crystalline egg albumin, of the solutions, and in the cleaning of the glassware. The egg albumin was made by Dr. L. Baker in this laboratory, crystallized three times, and dialyzed for 7 days. It was handled in a sterile way, throughout. Experiments were made at two different pH values. The solutions were all made in standard volumetric flasks, at constant temperature, and every piece of glassware was boiled for at least 2 hours in cleaning solution (sulfuric acid and sodium dichromate), then rinsed under distilled water, and, in the case of watch-glasses, flamed over a Bunsen burner, so as to obtain a perfectly "*wettable*" surface. The technique of these measurements has been dealt with at length in the twelve papers on the "Surface tension of serum" published in the Journal of Experimental Medicine, and especially in Paper X of this series.

The results of twenty-six sets of experiments, made by different experimenters with different batches of albumin, are given in Table I and Text-figs. 1 to 6. In Table I only the concentration at which the minima occurred is given. In the charts, the amplitude of the minimum can be obtained.

In order to make the results clearer, the occurrence of the minima was plotted against the concentration (see Text-fig. 7). In this chart the minima occurring at 1/85,000, 1/95,000, 1/135,000, and 1/145,000 were plotted as 1/80,000 to 1/90,000 and 1/140,000 for the sake of simplicity. However, they were taken into account for the calculation of the mean, with their real values.

*Interpretation.*—Three distinct groups of main minima are observed; the most frequent occurring around 1/140,000, another

TABLE I.
Concentration.

TABLE I.

| No. | pH | Concentration. 1/80,000 | 1/90,000 | 1/100,000 | 1/110,000 | 1/120,000 | 1/130,000 | 1/140,000 | 1/150,000 | 1/160,000 | 1/170,000 | 1/180,000 | 1/190,000 | 1/200,000 | 1/210,000 | 1/220,000 | 1/230,000 | 1/240,000 | 1/250,000 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 9 | 11 | 7 | 9 | 2 | 5 | 18 | 2 | 2 | 1 | 8 | 12 | 9 | 4 | 6 | 3 | 5 | 5 |
| | | First group. | | | | | Second group. | | | | | Third group. | | | | | | | |
| 1 | 4.9 | × | | × | | | | × | | | | | | | × | | | | |
| 2 | 4.9 | × | | × | | | | × | | | | | | | | | | | |
| 3 | 7.6 | | × | | × | | | | | | | | | | | | | | |
| 4 | 7.6 | × | | | | | | × | | | | | | × | | | | | |
| 5 | 7.6 | × | | | | | | | | | | | | | | | | | |
| 6 | 7.6 | | | | | | | | | | | | | | | | | | |
| 7 | 4.8 | | | × | | | × | | | | | × | | | | × | | | |
| 8 | 4.8 | × | | | × | | | × | | | | | × | | | | | | |
| 9 | 4.8 | × | | | × | | × | | | | | × | | | | | | | |
| 10 | 4.8 | × | | | × | | | | | | | | | | | | | | |
| 11 | 4.8 | | | × | | | | × | | | | | × | | | × | | | |
| 12 | 4.8 | | | | × | | | | | | | | | | | | | | |
| 13 | 7.6 | | × | | × | | × | | | × | | × | × | | | × | | | |
| 14 | 7.6 | | | × | | × | | | × | | | | | | | | | | |
| 15 | 7.6 | | | | × | | × | | | | | × | | | | | | | |
| 16 | 7.6 | | × | | | | | × | | | | × | | | | | × | | |
| 17 | 7.6 | | × | | | | | × | | | | × | | | × | | × | | |
| 18 | 7.6 | | × | | | | × | | | | | × | | | | | | | |
| 19 | 7.6 | × | | | | × | | × | | | | | | | | | | | |
| 20 | 7.6 | | × | | | × | | × | | | × | | | | | × | | | × |
| 21 | 7.6 | | × | | | | | × | | × | | | | | | | | | × |
| 22 | 7.6 | | × | | | | | × | | | | | | | | | | | × |
| 23 | 7.6 | × | | | × | | × | | | | | | | | | | | | |
| 24 | 7.6 | | × | | × | | | | | | | | | | | | | | |
| 25 | 4.9 | | × | × | | | | × | | | | | × | × | | | × | × | |
| 26 | 4.8 | | × | × | | | | × | | | | | × | × | | | | × | |

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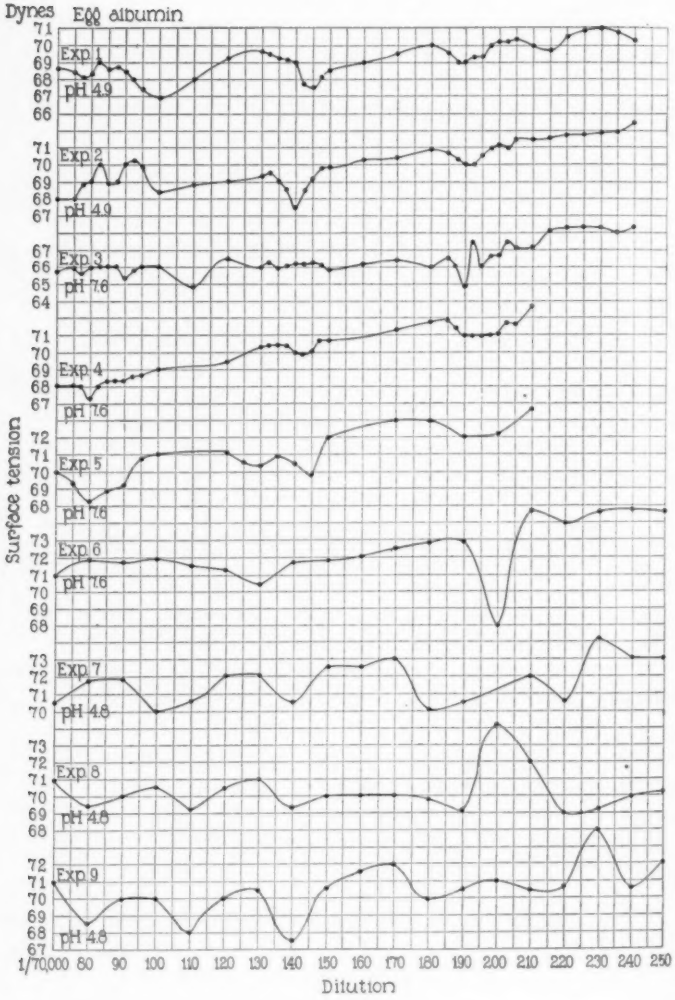
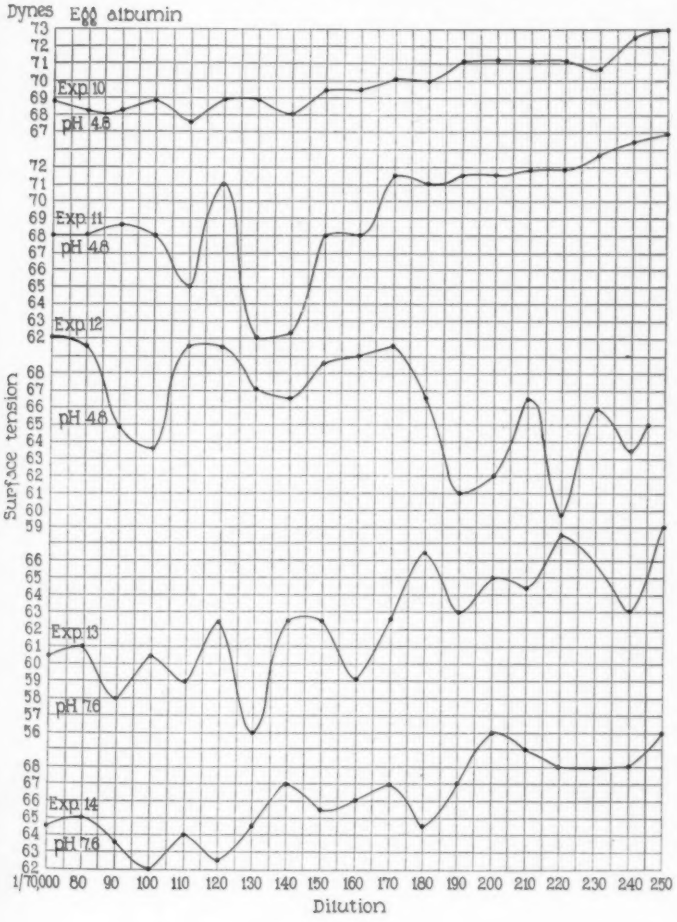

Dynes
Egg albumin
Exp. 1
pH 4.9
Exp. 2
pH 4.9
Exp. 3
pH 7.6
Exp. 4
pH 7.6
Exp. 5
pH 7.6
Exp. 6
pH 7.6
Exp. 7
pH 4.8
Exp. 8
pH 4.8
Exp. 9
pH 4.8
Surface tension
1/70,000 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250
Dilution

TEXT-FIG. 1.

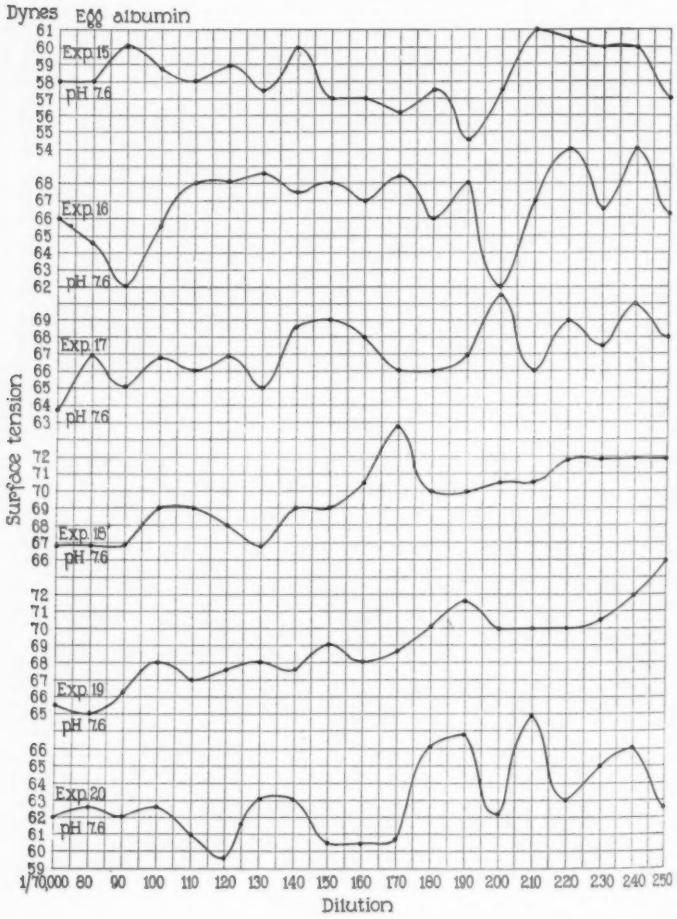
around 1/190,000, and the third, around 1/95,000. A fourth group is found to occur in certain experiments, around 1/240,000,




TEXT-FIG. 2.

but much less frequently. It is probably legitimate to arrange these minima into groups, because of the complicated structure of

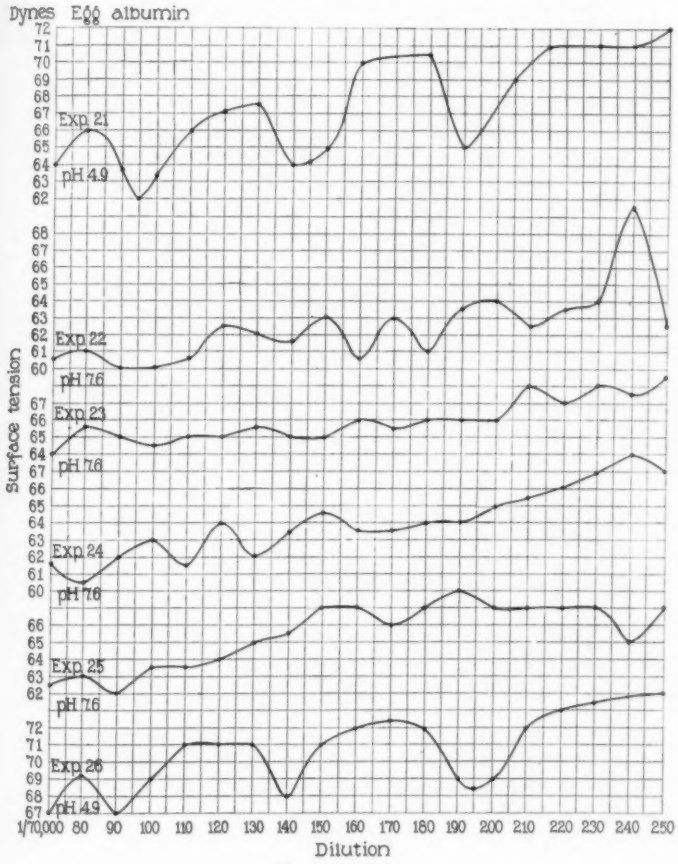
the albumin molecule which does not possess, as sodium oleate, one double bond and one $CH_3$ group which will always tend to




TEXT-FIG. 3.

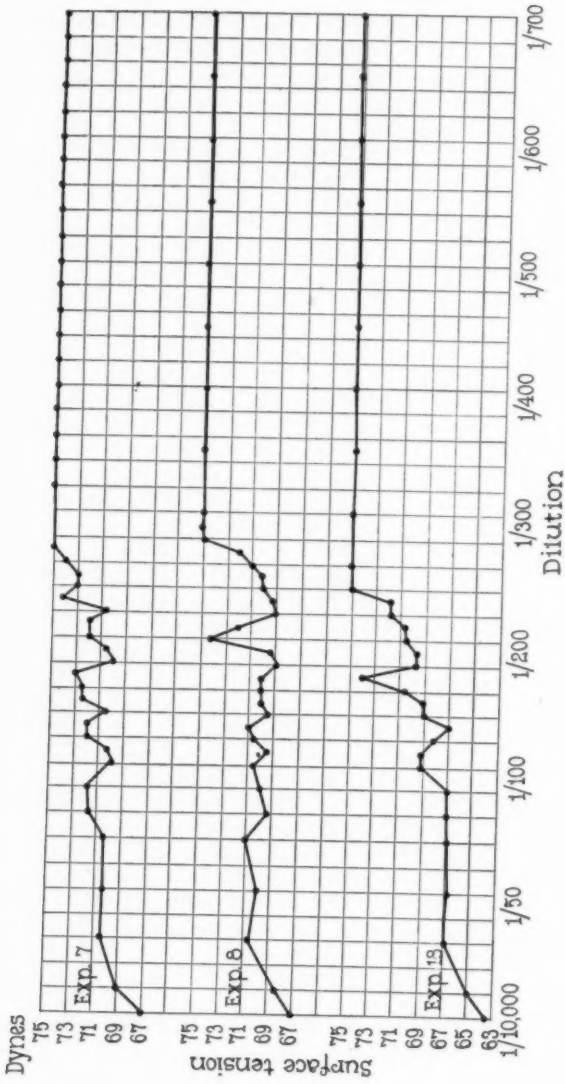
orient it in the same way. Instead of a double chain, bent at its middle, there may be a number of side chains, the behavior of

which, in solution, is quite unknown. Consequently, should it be admitted that a polarization is possible—and this view seems to us to be strongly supported by the constant sharp minima at

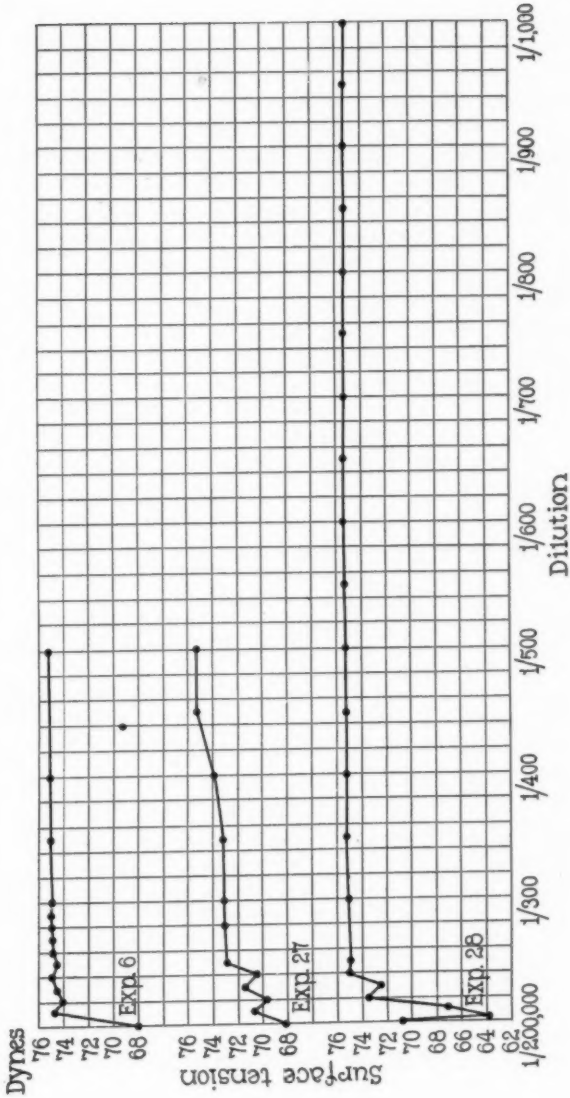



TEXT-FIG. 4.

1/140,000, which cannot be explained otherwise—the chances are that there may be a privileged axis of orientation in the molecule, corresponding to what might be termed its length. This


Surface tension
Dynes
75
73
71
69
67
Exp. 7
Exp. 8
Exp. 13
65
63
1/10,000
1/50
1/100
1/200
1/300
1/400
1/500
1/600
1/700
Dilution

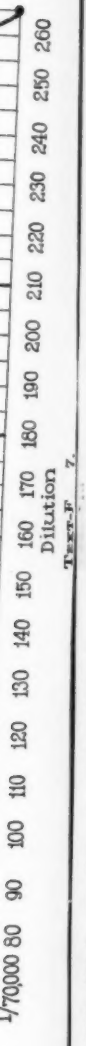
TEXT-FIG. 5.


Dynes
Surface tension
76
74
72
70
68
66
64
62
Exp. 6
Exp. 27
Exp. 26
1/200,000
1/300
1/400
1/500
1/600
1/700
1/800
1/900
1/1,000
Dilution

TEXT-FIG. 6.

Occurrence of minima
(26 experiments)
Per cent
77.0
58.0
38.5
19.2
3.85
20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1 0
1/70,000 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260
Dilution
1st group
Mean thickness
61.5 Å.u.
d = 19.9 Å.U.
d = 5
1/95,000
65.0
73.1
53.2
55.6
2nd group
Mean thickness
42.0 Å.u.
d = 5.9 Å.U.
1/139,000
41.7
45.0
29.0
39.1
3rd group
Mean thickness
30.3 Å.u.
d = 4.6 Å.U.
1/193,000
30.8
29.3
32.5
26.7
27.8
24.5
23.4
25.5

would explain the relative narrowness of the second group (Text-fig. 7) and the decided prevalence of minima immediately around 1/140,000. The width of the third group may be due to the fact that, when the molecules are horizontal, although there is a privileged orientation corresponding to the concentration 1/190,000, they can easily arrange themselves in four or five different ways, according to different planes of symmetry. In this case, all the horizontal molecules would not, as the rectangular prisms of sodium oleate, be oriented identically with reference to two vertical planes, but could rotate around their main horizontal axis. In other words, they could be considered provisionally, either as a series of prisms with a square base or as a series of polygonal prisms of irregular base with cross-sectional diameters varying from 32.5 to 27.9 Ångström units.[4] The mean value, taking into account the relative frequency of the minima, as expressed by the ordinates of Text-fig. 7, is 30.3 Ångström units. Although the second group seems to be more sharply defined than the third because the latter spreads over a relatively large range of concentrations (from




LEGEND FOR TEXT-FIG. 7.

TEXT-FIG. 7. In this chart, the frequency of occurrence of the minima of the value of static surface tension of crystalline egg albumin solutions at dilutions ranging from 1/70,000 to 1/250,000 is plotted as a curve. The figures of the abscissæ are the actual denominators divided by 1,000. This was done in order to avoid the crowding of the figures in a small space. The figures on the chart represent the thickness of the layer of adsorbed material in Ångström units, assuming that all the substance is adsorbed in conformity with the formula $L = \frac{MC}{A\delta}$, in which $L$ is the thickness; $C$, the concentration; and $M$, the mass of mixture in the watch-glass, always assumed at 22°C. = $2 \times 0.9979$ (temperature correction). This is true if the density of water is $\frac{1 \text{ gm.}}{\text{cm.}^3}$ and the concentration of the substance very small. $A$ = area of adsorbing surface (total surface of water in contact with air and glass = 26.4 sq. cm.) $\delta$ = specific gravity of substance in solution (1.295). The figures 1/95,000, 1/139,000, and 1/193,000 correspond to the mean value of the dilution at which the minimum occurs, the frequency of occurrence being taken into consideration for the calculation.

[4] The calculation of the thickness is done according to the formula given in the legend of Text-fig. 7. See also foot-notes 1 and 2.



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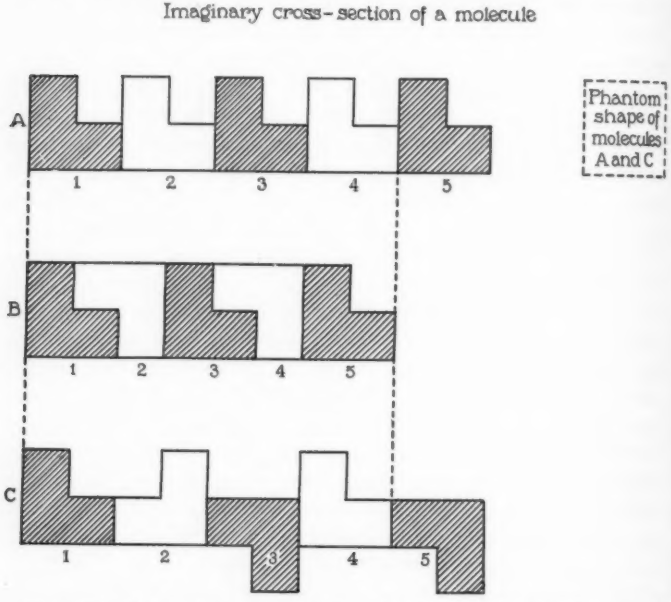
1/180,000 up to 1/210,000) the third covers a smaller range of thicknesses of the adsorbed layer than the second group from 1/130,000 to 1/150,000. The range in the second group is from 45 to 39.1 Ångström units, or a difference of 5.9 Ångström units. In the third group, the range is from 32.5 to 27.9 Ångström units, a difference of 4.6 Ångström units. Even by taking the minimum at 1/220,000 into account, as belonging to the third group, one obtains a difference of only 5.6, instead of 5.9 Ångström units. Consequently, the second and the third group are absolutely comparable, and the fluctuations of the minima can be attributed to less probable arrangements of molecules or to experimental errors. These two groups may be considered as expressing two dimensions of the molecule.

But when we consider the first group, although it looks identical with the third in Text-fig. 7 extending between the concentrations 1/80,000 and 1/110,000, we see that this range includes thicknesses varying between 73.1 and 53.2 Ångström units, the difference of which is 19.9 Ångström units. It is probable that we deal here with a composite layer 2 molecules thick. Indeed the mean value of the thickness if 61.5 Ångström units, which is about the double of the mean thickness of the third group, 30.3 Ångström units. Moreover, the three minima at 1/220,000, 1/200,000, 1/180,000, and sometimes that at 1/190,000, have their corresponding minima at exactly double the concentration, namely at 1/110,000, 1/100,000, 1/90,000, and 1/95,000, which means a layer twice as thick. And the minimum of the first group at 1/80,000 may correspond to a combination of one vertical and one horizontal layer. By adding the thickness of the monolayer at 1/140,000, namely 41.7 Ångström units, to that of the monolayers at 1/180,000 and 1/190,000 (respectively 32.5 and 30.8 Ångström units) one finds 74.2 in the first case, and 72.5 in the second, the mean value being 73.3, while the thickness of the layer at 1/80,000 is 73.1 Ångström units. Although the writer does not wish to lay emphasis on this interpretation given only tentatively, it seems to receive some support from the existence of a minimum at 1/70,000 which was observed in a few cases, and obviously corresponds to a double layer of vertical molecules, assuming the monolayer to be at 1/140,000.

Considering that on either side of this range, before 1/70,000

and beyond 1/250,000, no minima were observed, an attempt can be made to combine the data gathered in order to calculate the volume of the space occupied by 1 molecule. Of course, only the second and third groups of minima will be considered as it was assumed that the first corresponds probably to a series of double layers. Nothing is known concerning the shape of the molecules which appears to be quite complicated. If one assumes that each minimum expresses one dimension of the molecule, and one possibility of arrangement of molecules in a monolayer, one must admit, for the third group, at least four different diameters, the largest being 32.5 Ångström units, the smallest 27.3 Ångström units (between 1/180,000 and 1/220,000). But the difference in frequency of occurrence does not seem to support this view, and we shall first deal with another possibility. As a matter of fact, it is by no means certain that the molecule is prismatic in shape, with a polygonal base. We shall therefore admit, provisionally, that the space occupied by the individual molecule can be considered as prismatic, with a square base. This should give the maximum space which can be occupied by 1 molecule, and assumes that the values of the third group other than 1/190,000 are due to experimental errors. For the second group (length), there is a decidedly privileged axis of orientation, and a much greater probability of arrangement of molecules corresponding to a thickness of 41.7 Ångström units (at a concentration of 1/140,000), as seen in Text-fig. 7. Thus, for these two groups, it is assumed that the minimum occurring most frequently corresponds to the real thickness of the layer, and that the other less frequent minima are due to the lack of homogeneity of the solutions resulting in errors in the concentration. If this be the case the thickness at the concentration 1/190,000 is 30.8 Ångström units. The space occupied by 1 molecule is 39600 Å. u.$^3$ or $39.60 \times 10^{-21}$ cc. Its weight (the specific weight of the samples used in these experiments being 1.295) is $51.30 \times 10^{-21}$ gm. This is certainly the highest possible limit of the occupied space, and of the weight. The molecular weight would then be 30,800 (taking for $N$ the value $6.004 \times 10^{23}$). The molecular weight derived from chemical analysis is 15.703, which, multiplied by 2, equals 31,406. This figure differs by approximately 2 per cent from 30,800. Therefore, it seems logical to state that the factor 2 is correct.

In order to calculate the lowest probable limits (assuming that there is no interpenetration of the molecules) one must consider the space occupied by 1 molecule as delimited by a cylinder, the




TEXT-FIG. 8. Imaginary cross-section of molecules, showing three possible arrangements in a polarized monolayer. In the first row, *A*, as well as in the third row, *C*, the molecules do not interpenetrate each other, so that the "phantom cross-section" of each individual molecule, when surrounded by others, is a square. The "phantom shape" of such a molecule is a prism with a square base. In the second row, *B*, the molecules interpenetrate each other, so that 5 occupy the same volume as 4 of the first and third row. The square "phantom cross-section" does no longer exist, and the notion of specific gravity as characterizing the property of the "phantom shape" disappears. The apparent specific gravities in both cases are in the same ration as 4 to 5.

diameter of which is the mean value calculated above; namely, 30.3 Ångström units (corresponding to a concentration of 1/193,000, see Text-fig. 7). The volume of such a cylinder is 30300 Å. u.$^3$ or $30.30 \times 10^{-21}$ cc., and its weight $39.20 \times 10^{-21}$

gm. The molecular weight would be 23.550. Any other interpretations as to the shape of the molecule should forcibly yield a figure comprised between these two extreme limits.

DISCUSSION.

What may be the value of these figures? There is no doubt that it depends mainly on the correct interpretation of the minima and on the significance of the specific weight applied to 1 molecule. As this work is based on the previous experiments made with sodium oleate, which gave very accurate results,[3] the explanation of the minima, whether right or not, is not in question. On the contrary, even though it is admitted that our interpretation is correct on the whole, the figures can be questioned on the basis of a fundamental difference between the molecule of sodium oleate and that of egg albumin. The two single chains of the former were shown to occupy in space, when surrounded by similarly oriented molecules, a volume delimited by a parallelopiped. Hence, the specific gravity can be conceived, in such a case, as a property of all that space. The great accuracy of the figures obtained supported this view entirely. But in dealing with egg albumin, the same view is no longer forcibly correct. Supposing that there is, in certain cases, an interpenetration of the adjacent molecules, and that some minima are due, not to a difference in the dimensions, but to some special interlinking, then the space occupied by 2 molecules will no longer be equal to double the space occupied by one, and the notion of specific gravity, as given above, is meaningless. To take a crude example, suppose the cross-section of 1 molecule to be that of Text-fig. 8. Two sorts of arrangements are possible: (*a*) those which respect the "phantom shape" of the molecule, where no interpenetration takes place (*A* and *C*); (*b*) those which do not respect the "phantom shape," and interpenetrate, as in Text-fig. 8, *B*. In the first case, corresponding, for instance, to a concentration of 1/200,000 the specific gravity is a property of the whole "phantom shape," and the thickness is 29.3 Ångström units. In the other case (*B*), the concentration is increased in the ratio 5:4, the thickness would be calculated to be increased in the same ratio, while it is unchanged in reality. In other words, the specific gravity appears to have become 1.62, instead of 1.295. If such is the case for certain minima, the

TABLE II.

| I | II | III | IV | V | VI | VII | VIII |
|---|---|---|---|---|---|---|---|
| Substance. | Formula. | No. of C atoms. | Cross-section. | Length. | Volume of molecule. | Volume of 1 C atom = Column VI ÷ Column III. | Length per C atom. |
| | | | Å.u.$^2$ | Å.u. | Å.u.$^3$ | | Å.u. |
| Palmitic acid | $C_{15}H_{31}COOH$ | 16 | 21 | 24.0 | 508 | 31.8 | 1.5 |
| Stearic acid | $C_{17}H_{35}COOH$ | 18 | 22 | 25.0 | 552 | 31.2 | 1.39 |
| Cerotic acid | $C_{25}H_{51}COOH$ | 26 | 25 | 31.0 | 775 | 29.8 | 1.20 |
| Tristearin | $(C_{17}H_{35}O_2)_3C_3H_5$ | 57 | 66 | 25.0 | 1640 | 28.8 | 1.32 |
| Oleic acid | $C_{17}H_{33}COOH$ | 18 | 46 | 11.2 | 517 | 28.8 | 0.62 |
| Triolein | $(C_{17}H_{33}O_2)_3C_3H_5$ | 57 | 126 | 13.0 | 1630 | 28.6 | 0.69 |
| Trielaidin | $(C_{17}H_{33}O_2)_3C_3H_5$ | 57 | 120 | 13.6 | 1645 | 28.9 | 0.72 |
| Cetyl palmitate | $C_{15}H_{31}COOC_{16}H_{33}$ | 32 | 23 | 41.0 | 944 | 29.4 | 2.56 |
| Myricyl alcohol | $C_{30}H_{61}OH$ | 30 | 27 | 41.0 | 1118 | 37.0 | 1.37 |

calculations no longer hold true. In favor of the non-interpenetration, we have the experimental fact that some minima are formed at two concentrations the ratio of which is exactly 1:2, and also the satisfactory accord between the values of the molecular weight derived from chemical analysis (15,703 or an integer multiple of it)[5] and that obtained from the above calculations; namely, 30,800 as the upper limit. On account of the necessity of using the specific gravity as a factor, this figure would be quite different if the molecules could interpenetrate each other in different ways at different concentrations. In his remarkable work on the proteins,[6] Sörensen comes to the conclusion that the molecular weight is 34,000. He used a method based on osmotic pressure measurements of solutions at the isoelectric point. His higher value may be due to the fact that he had to use a solution relatively concentrated as compared with ours, and that some molecules may have been associated. However, it is interesting to remark that his experiments lead him to the same factor 2 for the value obtained from chemical analysis. Another relation can be established between the chemical composition, as given by Osborne, and the volume of the molecule obtained from our measurements. In order to establish it, it is necessary to refer to the dimensions of the molecules of nine organic compounds measured by Langmuir[7] and to calculate in all cases the volume occupied by 1 carbon atom in these molecules. The figures of Langmuir are given in Table II, Columns IV, V, and VIII. It will be clearly seen that, however different the molecules are in this series of nine, the volume occupied by 1 atom of carbon is very nearly the same in all cases but one, that of myricyl alcohol (Column VII), and show less discrepancies than the values for the length per carbon atom (Column VIII). If the figure for myricyl alcohol be eliminated as too exceptional, the mean value of the eight others, for the space occupied by 1 carbon atom is 29.7 Å. u.[3] The molecular weight 15,703 calls for 696 carbon atoms. It has been shown by Sörensen[6] and in this paper that this figure must be multiplied by 2 so that the molecule in solution is probably made of $696 \times 2 = 1{,}392$ atoms of carbon.

[5] Osborne, T. B., *J. Am. Chem. Soc.*, 1902, xxiv, 160.
[6] Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii.
[7] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

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The upper limit for the volume occupied by the albumin molecule, from our measurements being 39600 Å. u.³, the volume of 1 carbon atom can be obtained as for the substances of Table II by dividing 36,900 by 1,392. This should yield a figure whose agreement with the figures of Column VII (Table II) will either be a strong confirmation of all our statements or else condemn the method as unsound. The result is, for the volume occupied by 1 carbon atom in egg albumin 28.45 Å. u.³ which differs by only 4.4 per cent from the average 29.7, and by about 1 per cent from the value in four of the substances out of eight. It seems, therefore, that our assumptions were well founded. A certain number of facts are indeed required in order that the volume be that given as upper limit (39600 Å. u.³); namely, first, that the "phantom shape" of every individual molecule be that of a prism with a square base; and second, that the molecules do not interpenetrate so that the "density" is really a specific property of all the space delimited by the faces of this prism.

## CONCLUSIONS.

1. The tensiometric method of the minima of static surface tension was applied to crystalline egg albumin, and revealed the existence of numerous minima at dilutions between 1/70,000 and 1/250,000.

2. Some were much more frequently observed than others; namely, those at 1/90,000, 1/140,000, and 1/190,000. The less frequent minima occurring at and around 1/90,000 (namely at 1/80,000, 1/100,000, and 1/110,000) are considered as double layers of molecules, since, to every one of them corresponds another minimum at exactly double the dilution (1/160,000, 1/180,000, 1/200,000, and 1/220,000).

3. From the two principal minima, occurring most frequently, the dimensions of the "phantom shape" of the molecule are calculated. They are $41.7 \times 10^{-8}$ cm. and $30.8 \times 10^{-8}$ cm. Assuming the "phantom shape" to be a parallelopiped, and the specific gravity, a property of all the space occupied by it, it yields for the upper limit of the molecular weight 30,800. If mean values are taken into account, one obtains 30,000. This points to the factor 2 as that by which the molecular weight, derived from chemical analysis, should be multiplied.

4. Assuming that the shape is not that of a parallelopiped, and that it may be a prism with polygonal base, the lowest limit is given by considering it as a cylinder, the height of which is 42 Ångström units, and the diameter 30.3 Ångström units. It is then found to be 23,550. This hypothesis seems little probable.

5. From what is known of the probable molecular weight based on chemical analysis, the first figure appears to be more correct, as it corresponds almost exactly to twice the value derived from the knowledge of the sulfur content. This would seem to indicate that the molecules do not interpenetrate each other and that, when packed up in a similarly oriented way, they behave as though they occupied a "phantom space" in the shape of a parallelopiped with a square base. This view seems to receive some support from the fact that the mean space occupied by 1 atom of carbon in a series of eight organic compounds, is very nearly the same as that which it would occupy in the above described albumin molecule; namely, $28.45 \times 10^{-24}$ cc. Consequently, it is probable that the dimensions of the space occupied by 1 molecule of crystalline egg albumin, are $30.8 \times 30.8 \times 41.7$ Ångström units.

EXPLANATION OF PLATE 1.

FIG. 1. Apparatus for measuring the static value of the surface tension of series of solutions. The tensiometer is seen rolling on rails in front of the watch-glasses containing the solutions supported on a rigid table protected against vibrations.

FIG. 1.

(du Noüy: Crystalline egg albumin.)

XUM

# CHEMICAL CONSTITUENTS OF CAMEL'S URINE.

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(Received for publication, May 18, 1925.)

In experiments upon water hunger of the camel, *Camelus bactrianus*, undertaken in these laboratories[1] numerous observations extending over a period of several months were made upon the urine of this animal. These findings appear to be of general interest as an example of the peculiarities of the excretion of herbivorous animals.

A full grown animal was placed upon a standard diet consisting of sorghum leaves, sweet potato vines, and salt. 130 pounds of this dry fodder given daily were found adequate both from a nutritional and reproductive standpoint. The urine collected daily was carefully measured and estimations were made upon it for the following constituents: total nitrogen, ammonia, urea, creatinine, creatine, hippuric acid, chlorides, and the purine bases, as shown in Table I.

The urine had a dark, smoky, yellow color and smelt like sweet hay. It had a specific gravity from 1.045 to 1.056. The volume varied according to the time of observation. During the month of November an average of about 4,000 cc. *per diem* were obtained. During the colder weather this volume increased to more than 6 liters. As already reported when the animal was deprived of water for 10 days the volume of urine secreted fell to 1,800 cc.

The nitrogenous constituents of the urine are of interest. The camel excretes no ammonia and only the very slightest trace of urea was found present in the urine. The large amount of hippuric acid is similar, relative to the weight of the animal, to that found in the urine of cows and horses. (In three deter-

[1] Hawk, P. B., Practical physiological chemistry, Philadelphia, 8th edition, 1923.



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minations of the hippuric acid content of mules urine, an average of 1.08 per cent was obtained.)

A separate experiment was undertaken to identify the character of the organic acid considered to be hippuric acid. A specimen was prepared by Roaf's method which when decolorized and recrystallized five times from water, yielded a product giving the characteristic melting point, nitrogen content, and other properties of hippuric acid.

TABLE I.

*Analysis of 24 Hour Camel's Urine.*

Female, weight 1,129 lbs.

| | *gm.* | *gm. N* | *per cent of total N* |
|---|---|---|---|
| Total N* | 8.70 | | |
| Ammonia* | 0 | 0.0 | 0 |
| Urea* | Very slight trace. | | 0 |
| Total creatinine* | 9.24 | 3.43 | 39.4 |
| Creatine† | 3.97 | 1.27 | |
| Hippuric acid‡ | 39.00 | 3.05 | 35.1 |
| Purine bases§ | 4.70 | 1.73 | 19.9 |
| Chlorides¶ | 7.99 | | |
| Total | | | 94.4 |

Averages of 1 month's estimations conducted under standard dietary conditions, by standard methods (Read, B. E., *China Med. J.*, 1920, Anat. Suppl., xxxiv, 18).

* Folin.

† Folin-Benedict-Myers.

‡ Folin-Flanders.

§ Walker.

¶ Volhard-Harvey.

While hippuric acid is not considered a direct measure of protein metabolism it is readily seen that on such a limited diet, this acid is a fair measure of exogenous metabolism. On the other hand, the high values obtained for creatinine and creatine show an endogenous metabolism comparable to that of man. The total purine bases, while not examined in detail for their content of allantoin, uric acid, etc., are similar in relative amounts to those obtained from other animals. The same is true for chlorides and sulfates.

The animal was pregnant at the time of the above determinations. One month after parturition, during the lactation period, estimations were again made of the total nitrogen and hippuric acid in the urine. An average of 118.2 gm. of hippuric acid and 22.68 gm. of total nitrogen was found on 3 consecutive days.

Throughout North China on the many narrow roads which lead the traveller for hundreds of miles, there are to be found, about every quarter of a mile, straw beds for the collection of animals' urine. The camels, donkeys, and other beasts of burden, which pass along the highways, are induced by the odor to urinate at these places. Seeing that the urine when fresh contains no ammonia, it would be of particular interest to investigate the nature of the enzyme which sets up ammoniacal fermentation of the hippuric acid present. These straw beds, when considered sufficiently mature, are dug up and greatly prized as fertilizer. This procedure suggests other investigations associated with the oxidative processes involved.

# THE OXIDATION OF ISOBARBITURIC ACID.

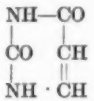
## A NEW CLASS OF INDIGOÏDS.

By DAVID DAVIDSON and OSKAR BAUDISCH.

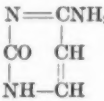
*(From the Laboratories of The Rockefeller Institute for Medical Research.)*

(Received for publication, May 21, 1925.)

In studying the oxidation of natural occurring pyrimidines, Baudisch (1) observed that uracil (I) and cytosine (II) yielded solutions which were brilliant yellow in color. The coloring agent, or agents, were, however, not isolated. Since isobarbituric acid (III) could be demonstrated to be an intermediate product in the oxidation of these pyrimidines, it was anticipated that an investigation of this substance might give an insight into the nature of the colored derivatives.



I.



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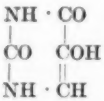

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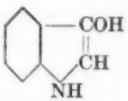
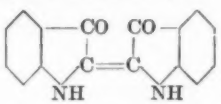


IV.

The subject appeared especially promising because of the possible analogy between isobarbituric acid (III) and indoxyl (IV). It was to be expected that just as the latter yields indigo (V) on oxidation, so isobarbituric acid might give rise to a highly colored bicyclic molecule (VI).



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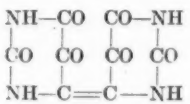
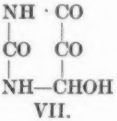


VI.

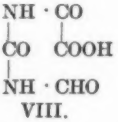
Previous investigators have shown that on oxidation isobarbituric acid is readily attacked at the double bond. Thus, by bromine water[1] it is converted into isodialuric acid (VII), while

[1] Behrend and Roosen (2), p. 242.

with permanganate the cycle is ruptured and formyl-oxalyl-urea (VIII) is produced (3).

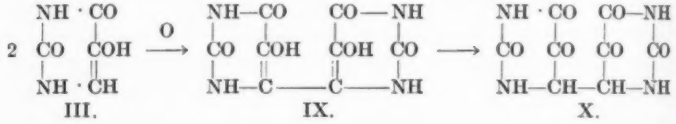


VII.



VIII.

By utilizing ferricyanide as the oxidant, it has now been found possible to control the oxidation so that, in a portion of the oxidized material at least, the double bond remains intact, the reaction proceeding with the loss of 1 hydrogen atom from the molecule and the subsequent coupling of two cycles (formula (IX)).

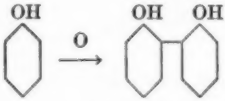


III. IX. X.

For this product the name 4.4′-di-isobarbituric acid is proposed. The compound is of a bright lemon-yellow color, is exceedingly insoluble, forms red alkali metal salts, and dissolves in excess of alkali to give solutions possessing a beautiful green fluorescence. Since, unlike isobarbituric acid, and other 5-hydroxypyrimidines, it does not yield a blue salt with ferric chloride, it probably exists in the keto form (formula (X)).

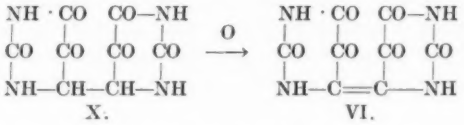
*Urindigo.*

Thus far the expected analogy between isobarbituric acid and indoxyl had not been realized, the behavior of isobarbituric acid resembling much more that of aromatic phenols which, on oxidation, yield ortho-diphenols,



with this difference, however, that di-isobarbituric acid no longer retained the enol form (IX), but appeared rather in the keto form (X). It seemed probable, therefore, that further oxidation

of di-isobarbituric acid might lead to the unsaturated derivative (VI) originally anticipated. This was found to be the case, for with nitric acid or bromine water di-isobarbituric acid is converted into a scarlet-colored compound with the loss of 2 hydrogen atoms.



X. VI.

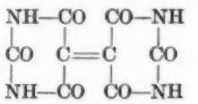
This substance contains the indigoïd grouping $-CO-C=C-CO-$, hence its systematic name (4) is di-uracil-4.4′-indigo. It is the first colored representative of the pyrimidine indigoïds.[2] Because of its close similarity to indigo (V) and its derivation from uracil, the convenient name "urindigo" is proposed for it.

## EXPERIMENTAL PART.

*Isobarbituric Acid.*—A modification of the method of Behrend and Roosen[3] was employed. 50 gm. of 5-nitro-uracil (5) and 100 gm. of tin were heated on the steam bath with 550 cc. of water and 550 cc. of concentrated hydrochloric acid until all the nitro compound had gone into solution and hydrogen was being evolved. The reaction mixture was then quickly filtered by suction. The filtrate, on cooling, yielded 26 gm. of pure, colorless isobarbituric acid (63 per cent of theory). About 5 gm. of 5-amino-uracil may be recovered from the mother liquor.

*Di-Isobarbituric Acid.*—A chilled (5°) filtered solution of 51.2 gm. (2 mols) of potassium ferricyanide in 200 cc. of water is

[2] The isomeric colorless dehydro-hydurilic acid



has been described by Biltz and Heyn (Biltz, H., and Heyn, M., *Ber. chem. Ges.*, 1919, lii, 1306) and its tetramethyl derivative by Biltz, Heyn, and Hamburger (Biltz, H., Heyn, M., and Hamburger, T., *Ber. chem. Ges.*, 1916, xlix, 662).

[3] Behrend and Roosen (2), p. 239.

added at once to a chilled, filtered solution of 10 gm. (1 mol) of isobarbituric acid, and 13.2 gm. (3 mols) of potassium hydroxide in 200 cc. of water. The reaction mixture is at first dark brown and opaque, but after being stirred a few moments, it becomes bright red and transparent, whereupon the red dipotassium salt of di-isobarbituric acid begins to precipitate. The separation of the product is facilitated by shaking the reaction mixture for a few minutes. After centrifugalizing, the salt is suspended in water, filtered, and washed with water. Yield 1.5 gm.[4]

*Analysis.*

| | | |
|---|---|---|
| $C_8H_4O_6N_4K_2$. | Calculated. | N 16.97. |
| | Found. | " 17.12. |

That the filtrate contains isodialuric acid (or dialuric acid) is shown by the precipitation of a purple salt on adding barium hydroxide.

To obtain the free di-isobarbituric acid the salt was suspended in water and poured into 200 cc. of boiling water containing an excess of acetic acid. The very insoluble lemon-yellow product separated immediately in microscopic rhombic plates, was filtered hot, and washed with hot water.

*Analysis.*

| | | |
|---|---|---|
| $C_8H_6O_6N_4$. | Calculated. | C 37.78, H 2.38, N 22.06. |
| | Found. | " 38.06, " 2.64, " 22.17. |

Di-isobarbituric acid is very insoluble in all common solvents. It dissolves in concentrated sulfuric acid, and is reprecipitated on dilution. When treated with dilute alkali, it is converted into its red alkali salts, which dissolve in excess of alkali to give solutions possessing a strong green flourescence. The red alkali salts may be reprecipitated from these solutions by passing in carbon dioxide. Di-isobarbituric acid reduces silver nitrate solution in the cold. With concentrated nitric acid it gives a brilliant scarlet solution (urindigo), the color of which gradually fades. On evaporation of the solution and moistening the residue with ammonia, a deep purple-red color is obtained (murexide

[4] It is to be noted that a small quantity of the red sodium salt of di-isobarbituric acid is obtained on evaporating a solution of isobarbituric acid in sodium bicarbonate in an open dish on the water bath, or by passing oxygen into a warm solution of sodium isobarbiturate.

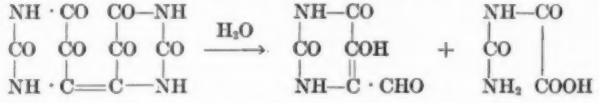
test). With diazobenzenesulfonic acid and sodium bicarbonate it gives no immediate color, but gradually develops a deep wine color on standing. It does not melt below 310°.

*Urindigo.*—1 gm. (1 mol) of finely pulverized di-isobarbituric acid was suspended in about 50 cc. of cold water and 0.2 cc. (1 mol) of bromine added, and the mixture vigorously shaken. The yellow acid was immediately transformed into the scarlet indigoïd, which was filtered off and washed with water. Yield 0.7 gm. The product was bromine-free.

*Analysis.*

| | | |
|---|---|---|
| $C_8H_4O_6N_4$. | Calculated. | C 38.08, H 1.60, N 22.23. |
| | Found. | " 38.09, " 1.87, " 21.95. |

Urindigo is insoluble in the common organic solvents. It dissolves in concentrated sulfuric acid, from which it is reprecipitated on dilution. It dissolves in concentrated nitric acid, but is gradually oxidized. It slowly dissolves in bromine water; if the solution is now treated with excess of barium hydroxide, a purple precipitate (barium dialurate) is obtained. It is immediately decomposed by alkali; on neutralizing the solution and adding ferric chloride, the deep blue ferric salt characteristic of 5-hydroxy derivatives of uracil is obtained. The solution probably contains 5-hydroxyuracil-4-aldehyde, the urindigo presumably having undergone a type of hydrolysis peculiar to indigoïds (6).



Urindigo does not melt below 310°.

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# THE REDUCING SUBSTANCES OF THE BLOOD.

By ALMA HILLER, G. C. LINDER, and D. D. VAN SLYKE.

*(From the Hospital of The Rockefeller Institute for Medical Research.)*

(Received for publication, May 5, 1925.)

The occurrence, in many cases of advanced nephritis, of high blood sugar values, as determined by the various reduction methods, has been repeatedly confirmed (1). It is known that none of the reduction methods is entirely specific for glucose. We have consequently controlled the results in a number of such cases by determining the fermentability of the reducing substance, and also its ability to disappear under the influence of the zymase of the blood itself.

In an attempt to find a method which would be more specific for glucose, the older workers turned to polariscopic and fermentation methods. Maase and Tachau (2) found good agreement between polariscopic determinations and the reduction methods, using both the copper reduction method of Bertrand, and the mercury reduction method of Tachau, on normal human blood and blood with high sugar values. They concluded that all the reducing substance in the blood was present in the form of glucose. Frank and Bretschneider (3) compared the reducing value of blood by the Bertrand copper method before and after fermentation with yeast. They found that human blood after fermentation contained no reducing substances. In adrenalin hyperglycemia in dogs and in rabbits they found 0.01 to 0.03 per cent of non-fermenting reducing substances. Mayer (4) found non-fermenting reducing values by the method of Fehling. Griesbach and Strassner (5) found no non-fermenting reducing values in the blood by the Lehmann-Maquenne method, and also found polariscopic and reduction values to agree. Lyttkens and Sandgren (6) found in twelve human blood samples after fermentation 0.03 to 0.053 per cent of reducing substances by the Bang method. Schumm (7) performed similar experiments, using various reduction methods. He found no reducing value after fermentation when using the Bertrand method, but found reduction by the Lehmann-Maquenne method. Schumm and Hegler (8) found an average of 0.036 per cent of reducing substances after fermentation in three samples of normal human blood by the Bang method, and 0.0037 per cent by the Bertrand method. Non-fermentable reducing substances from 76 patients,



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covering a wide range of diseases, were equivalent to an average of 0.039 per cent of glucose by the Bang method. Three nephritic bloods ranged from 0.03 to 0.04 per cent. Schumm concluded that the amount of non-glucose reducing substance found was dependent upon the reduction method used. Stepp (9–12) compared polariscopic and fermentation methods with the reduction methods of Bertrand and Lehmann-Maquenne. He found that the polariscopic and the fermentation methods agreed well, and averaged 20 to 30 per cent, sometimes even 50 per cent lower than the reduction figure, both in normal bloods and in the blood of diabetic and nephritic patients. He found that in diabetes the higher the blood sugar the greater was the difference between reduction and polariscopic determinations. The difference ranged from 0.046 to 0.141 per cent calculated as glucose (9). In nephritis the difference was less significant, varying from 0.021 to 0.096 per cent, while the normals varied from 0 to 0.062 per cent (10). Stepp (12, 13) also used the unique method of determining the carbon content of the protein-free filtrate, after proteins, purines, etc., had been removed with phosphotungstic acid. He then calculated the amount of carbon which would be due to glucose, according to the reduction values, and compared this value with the total carbon value. The diabetic bloods on which he performed the experiments showed variation in results; some showed extra carbon, others did not. Ege (14) found the non-fermenting reducing values in blood amounting to only 0.002 to 0.009 per cent by Bang's micro method. He stated (15) that by Bang's micro blood sugar method as modified by Ege, and by the method of Hagedorn and Jensen (16) this residual reduction is so slight that in normal persons it has no practical clinical importance. In uremic patients, however, he found that the residual reduction might be great. Criticisms of the polariscopic and fermentation methods have been adequately set forth by Macleod (17) and by Höst and Hatlehol (18), who have also compared the reduction methods which are most commonly used at present.

The various authors constantly emphasize the dependence of results upon the reduction method used.

It is apparent from the literature that there may be a definitely determinable, though small, amount of reducing substances in the blood, which cannot be attributed to glucose. The exact nature of these substances is not definitely known, although it is known that uric acid, creatinine, creatine, glucuronic acid, pentoses, disaccharides, purines, and adrenalin have the power of reducing the reagents commonly used in blood sugar determinations.

We have attempted to determine this non-glucose reducing fraction in nephritic bloods with both high and normal total reducing values, and to relate these values to the products of nitrogen

retention. The methods were also applied to the blood of rabbits under the influence of insulin, to discover the level of the actual glucose value during insulin hypoglycemia.

## EXPERIMENTAL.

### *Methods.*

The reducing substances in whole blood were determined chiefly by the method of Hagedorn and Jensen (16). The methods of Shaffer and Hartmann (19), Folin and Wu (20, 21), and Myers and Bailey (22) were compared with the method of Hagedorn and Jensen. Each of these methods yields results constant within about 0.005 per cent of glucose. The reagents for each method were standardized by analyses of standard glucose solutions, and curves were constructed showing the amounts of glucose indicated by the varying amounts of reduction.

*Total Reducing Substances.*—Freshly drawn blood was either defibrinated or rendered non-coagulable by addition of 0.2 per cent of potassium oxalate. The reducing value was estimated in terms of glucose.

*Residual or Non-Glucose Reducing Substances.*—Two methods of removing glucose from the blood were used.

In the *fermentation method* the glucose was removed by fermentation with yeast. To 1 cc. of blood was added 0.5 cc. of a freshly prepared suspension of Fleischmann's compressed yeast, prepared by suspending one-fourth cake in water to a total volume of 5 cc. The blood was incubated at 38°C. Samples were removed at the end of 20 minutes for determination of the reducing substances. Blank determinations on the yeast were run along with the blood, 1 cc. of distilled water being substituted for the 1 cc. of blood. After the yeast blank had been subtracted the reducing substance determined was calculated as the "residual reducing substances" in terms of glucose.

In the *glycolysis method* the glucose was removed by means of the glycolytic power of the blood itself. In most instances the blood was defibrinated, but in a few cases coagulation was prohibited by the addition of 0.2 per cent of potassium oxalate. 1 cc. of blood was placed in a small test-tube, 1.5 by 6 or 8 cm., tightly stoppered with a rubber stopper, suspended in a water bath at

38°C. (23, 24), and kept in motion at a slow rate, in order to keep the cells in contact with the plasma, since, according to Milne and Peters (25), and Macleod (17, 24), glycolytic activity is an intracorpuscular process. Samples of blood were taken after 24 and 48 hours for determination of the reducing power. This gave the residual reducing substances in terms of glucose. Glycolysis was in nearly all cases complete in 24 hours, as was also reported by Bürger (23).

According to Hahn (26) oxalate does not check glycolysis. According to Macleod (17) glycolysis is interfered with by concentrations over 0.1 per cent, while Rubino and Varela (27) found that concentrations up to 0.32 per cent have no effect on glycolysis. Evans (28) reported that 1 per cent of sodium fluoride or potassium oxalate inhibits glycolysis completely, while 0.2 per cent of potassium oxalate retards the rate slightly. In two experiments we have used blood with 0.2 per cent of oxalate, in the remainder defibrinated blood. Most, but not all, of the experiments were carried on under aseptic conditions. Cajori and Crouter (29) and Tolstoi (30) did not find glycolysis increased in blood samples which showed positive cultures in the course of the experiment.

*The Non-Protein Nitrogen of the Plasma* was determined by the method of Folin and Wu (20), with the Bock and Benedict technique for micro distillation (31) and with 0.01 N solutions of HCl and NaOH for titration. *Urea Nitrogen* was determined on whole blood by the method of Van Slyke and Cullen (32). *Uric Acid* was determined on whole blood by the method of Benedict (33). *Creatinine* determinations were made on whole blood by the method of Folin and Wu (20). *Serum Phosphorus* was determined by the method of Tisdall (34). *Hydrogen Ion Concentration* was determined by the micro method of Hastings and Sendroy (35).

*Comparison of Methods for Determining Blood Sugar over a Low Range of Concentration.*

The colorimetric methods of Folin and Wu (20, 21) and Myers and Bailey (22), and the titrimetric method of Shaffer and Hartmann (19) were compared with the titrimetric method of Hagedorn and Jensen (16) before and after glycolysis. The Hage-

dorn and Jensen method was applied also to the Folin and Wu tungstic acid filtrate. The results are recorded in Table I.

The picric acid method of Myers and Bailey gave the highest results after 24 hours glycolysis. This figure was higher than that of a 4 hour glycolysis period in both experiments. It appeared that during prolonged incubation of blood some substance or substances were formed which reduced picric acid. The Folin-Wu, Hagedorn-Jensen, and Shaffer-Hartmann methods gave sufficiently concordant results for residual reducing substances, when the latter exceeded 0.02 per cent.

TABLE I.

*Comparison of Methods for Determining Small Amounts of Reducing Substances after Glycolysis of Defibrinated Dog Blood.*

| Sample No. | Time of incubation at 38°C. | Method. | | | | | Tungstic acid filtrate concentrated to 1/5 volume. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Hagedorn and Jensen. | Hagedorn and Jensen on tungstic filtrate. | Folin and Wu. | Myers and Bailey. | Shaffer and Hartmann. | Hagedorn and Jensen. | Folin and Wu. | Shaffer and Hartmann. |
| | *hrs.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 1 | 0 | 0.102 | 0.102 | 0.093 | 0.109 | | | | |
| | 4 | 0.028 | 0.020 | 0.020 | 0.018 | 0.029 | | | |
| | 24 | 0.023 | 0.013 | 0.018 | 0.026 | 0.023 | | | |
| 2 | 0 | 0.093 | 0.095 | 0.099 | 0.102 | 0.115 | | | |
| | 4 | 0.020 | 0.024 | 0.024 | 0.015 | 0.026 | 0.036 | 0.021 | 0.033 |
| | 24 | 0.020 | 0.017 | 0.015 | 0.026 | 0 | 0.025 | 0.012 | 0.017 |

For glucose concentrations below this figure the Shaffer-Hartmann method is not applicable, as it fails to show any reduction. The other two methods agreed throughout. The Hagedorn-Jensen titration could be used on the Folin-Wu filtrate, as well as on the filtrate prepared according to Hagedorn and Jensen.

When the tungstic acid filtrate from the glycolyzed blood sample was concentrated *in vacuo* to one-fifth of its volume, and the reducing power was then determined, the value by the Shaffer and Hartmann method was found to agree, within experimental error, with the other methods. It seems, then, that the apparent negative result obtained by the Shaffer and Hartmann method is due rather to a lack of oxidizing power of the reagent when

very low concentrations of these reducing substances are present, than to the absence of reducing substances.

We have used the Hagedorn and Jensen method for the estimation of reducing substances in all of the following experiments.

*Residual Reductions after Fermentation and Glycolysis.*

In preliminary fermentation experiments the blood samples were incubated with yeast as above described for periods of 20 minutes and 2 hours. The results are shown in Table II. Fermentation of the sugar was apparently complete in 20 minutes. There was a slightly higher value for reducing substances present at the end of 2 hours than at the end of 20 minutes incubation. This was repeatedly observed. Apparently secondary reactions in the yeast-blood mixture result in a slow formation of reducing substance. This phenomenon has been pointed out by Höst and Hatlehol (18) and others. To judge its rate from comparison of the 20 minute and 2 hour analyses, the reducing substance thus formed in the 20 minute period was not significant.

Glycolysis yielded about the same residual reductions as the 20 minute fermentation. It is apparent that the amount of reducing substance which disappears spontaneously when blood is incubated for 24 hours is a definite quantity, leaving a residual reduction which does not change on longer incubation. We feel justified, therefore, in concluding that at the end of 24 hours, glycolysis had ceased, and that the residual value is due to substances other than free glucose. In one experiment, Table V, where the residual value was high after glycolysis, 0.084 per cent, the blood was fermented with yeast to see if any further decrease could be accomplished. The value after fermentation was practically unchanged; *viz.*, 0.088 per cent.

*Glycolysis of Glucose Added to Blood.*—In order to test the glycolytic action of the blood on glucose, varying quantities were added to blood, which was then incubated at 38°. The results of such an experiment are shown in Table III. It is apparent that regardless of the amount of glucose present, within physiologically occurring limits, incubation destroys it and leaves a definite residue of non-glucose reducing substances. In the sample with the larger glucose addition 48 hours were required

TABLE II.

*Comparison of Fermentation and Glycolysis Methods for Removing Glucose from Blood.*

| Blood sample. | Method of removal of glucose. | Time of incubation. | Reducing substances as glucose. |
|---|---|---|---|
| | | | *per cent* |
| Rabbit. | | 0 | 0.099 |
| | Yeast fermentation. | 20 min. | 0.025 |
| | " " | 120 " | 0.042 |
| Human. | | 0 | 0.106 |
| | " " | 20 min. | 0.012 |
| | " " | 120 " | 0.025 |
| | Glycolysis. | 24 hrs. | 0.019 |
| | " | 48 " | 0.019 |
| Human. | | 0 | 0.090 |
| | Yeast fermentation. | 20 min. | 0.011 |
| | Glycolysis. | 24 hrs. | 0.011 |
| | " | 48 " | 0.011 |

TABLE III.

*Glycolytic Power of a Nephritic Blood in Which Glucose Has Been Added.*

| Sample. | Reducing value in terms of glucose. | | |
|---|---|---|---|
| | Before incubation. | After 24 hrs. incubation. | After 48 hrs. incubation. |
| | *per cent* | *per cent* | *per cent* |
| Blood alone. | 0.117 | 0.070 | 0.079 |
| " + glucose. | 0.213 | 0.073 | 0.083 |
| " + " | 0.343 | 0.150 | 0.077 |

TABLE IV.

*Effect of pH on Glycolysis, within the Range of pH Variation Found during the Process.*

| Subject. | pH | | Reducing substances estimated as glucose. | |
|---|---|---|---|---|
| | Before glycolysis. | After 24 hrs. glycolysis. | Before glycolysis. | After 24 hrs. glycolysis. |
| | | | *per cent* | *per cent* |
| M. R. | 7.61 | 7.30 | 0.086 | 0.030 |
| M. R. + lactic acid | 7.23 | 7.05 | 0.088 | 0.027 |

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to complete the destruction, but the final residual reduction was the same as in the other samples.

*Effect of pH Change on Completeness of Glycolysis.*—The fact that increased acidity retards glycolysis in blood has been repeatedly observed (36, 37, 38, 24). In order to determine whether the acid produced during glycolysis could act as a limiting factor of the reaction we added to blood amounts of lactic acid (0.15 cc. of 0.155 N acid to 3 cc. of blood) equivalent to the amount that would be formed by complete conversion of glucose into this acid. The fall in pH thus caused had no apparent effect on the completeness of the glycolysis. Such an experiment is recorded in Table IV. The final low pH reached at the end of glycolysis, 7.05, as the result of added acid plus that formed by the glycolysis, was lower than any observed after glycolysis alone.

*Insulin Experiments.*—The blood of rabbits was examined during insulin convulsions by the glycolysis method to determine whether insulin was capable of removing all the glycolyzable substances from the blood. Rabbits which had fasted for 24 hours were used for these experiments. Blood was removed as aseptically as possible from the ear vein, dropped into sterile tubes, and defibrinated. The reducing substances were determined immediately and after 24 and 48 hours of incubation at 38°C. After the preliminary blood samples were drawn the animals were injected subcutaneously with large doses of insulin (10 or 20 units of Iletin, Lilly). At the time of the appearance of convulsions samples of blood were again removed from the ear vein as described above, and reducing substances were determined in the same way, before and after incubation. The results are shown in Table V.

*Reducing Values of Creatinine and Uric Acid by the Hagedorn and Jensen Method.*—Solutions of uric acid and of creatinine, and mixed solutions of various strengths, as well as mixtures with blood, were made up, and the reducing values determined. The pH in each case was adjusted to neutrality. The composition of the solutions as well as the results obtained are recorded in Table VI.

*Estimation of Reducing Substances of the Blood in Normal Individuals and in Nephritics.*—Table VII gives determinations of total and residual reducing substances in a number of nor-

TABLE V.

*Effect of Insulin on the Reducing Substances in the Blood of Rabbits.*

| Rabbit No. | Time of glycolysis. | Reducing substances calculated as glucose. | |
|---|---|---|---|
| | | Before insulin. | During convulsions. |
| | *hrs.* | *per cent* | *per cent* |
| 7 | 0 | 0.133 | 0.060 |
| | 24 | 0.084 | 0.048 |
| | 48 | 0.081 | 0.049 |
| | Fermentation. | 0.088 | |
| 8 | 0 | 0.133 | 0.054 |
| | 24 | 0.034 | 0.046 |
| | 48 | 0.029 | 0.050 |
| 9 | 0 | 0.121 | 0.066 |
| | 24 | 0.065 | 0.051 |
| | 48 | 0.063 | 0.049 |
| 766 | 0 | 0.118 | 0.036 |
| | 24 | 0.036 | 0.030 |
| | 48 | | 0.032 |
| 778 | 0 | 0.139 | 0.056 |
| | 24 | 0.031 | 0.029 |
| | 48 | | |
| 745 | 0 | 0.118 | 0.073 |
| | 24 | | 0.022 |

TABLE VI.

*Reducing Value of Creatinine and Uric Acid by the Hagedorn and Jensen Method.*

| Solution No. | Composition of solution. | | | Reducing value found in terms of glucose. | Reducing value calculated. | Remarks. |
|---|---|---|---|---|---|---|
| | Uric acid. | Creatinine. | Total volume. | | | |
| | *mg.* | *mg.* | *cc.* | *per cent* | *per cent* | |
| 1 | 2 | 2 | 100 | 0.001 | | Amounts in normal blood. |
| 2 | 9.4 | | 100 | 0.001 | | |
| 3 | | 15.6 | 100 | 0.009 | | |
| 4 | 9.4 | 15.6 | 100 | 0.013 | | Amounts in blood in the high N retention. |
| 5 | | | | 0.073 | | Normal blood. |
| 6 | 4.7 | 7.8 | 100 | 0.044 | 0.043 | 1:1 mixture of Solution 4 and Blood 5. |

TABLE VII.

*Reducing Substances in the Blood of Normal Subjects and of Nephritics.*

| Subject. | Diagnosis. | Date. | Total reducing substances calculated as glucose. | Residual reducing substances calculated as glucose. | Glucose by difference. | Method of removing glucose. | pH | Serum inorganic P per liter. | Plasma total non-protein N per 100 cc. | Urea N per 100 cc. | Uric acid per 100 cc. | Creatinine per 100 cc. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *per cent* | *per cent* | *per cent* | | | *mM* | *mg.* | *mg.* | *mg.* | *mg.* |
| Dog 1. | Normal. | Jan. 14, 1924 | 0.095 | 0.035 | 0.060 | Glycolysis. | | | | | | |
| " 2. | " | " 22, 1924 | 0.102 | 0.023 | 0.079 | " | | | | | | |
| " 3. | " | Feb. 14, 1924 | 0.093 | 0.020 | 0.073 | " | | | | | | |
| " 4. | " | Mar. 18, 1924 | 0.106 | 0.019 | 0.087 | " | | | | | | |
| Horse. | " | | 0.088 | 0.017 | 0.071 | Fermentation. | | | | | | |
| G. L. | Normal. | Jan. 17, 1924 | 0.083 | 0.017 | 0.066 | Glycolysis. | | | | | | |
| G. L. | " | " 6, 1924 | 0.098 | 0.032 | 0.066 | Fermentation. | | | | | | |
| J. M. | " | " 8, 1924 | 0.105 | 0.023 | 0.082 | Glycolysis. | 7.40 | 1.30 | 25 | 12 | 2.0 | 1.3 |
| B. B. | Chronic nephrosis. | Apr. 15, 1924 | 0.090 | 0.011 | 0.079 | Glycolysis. | 7.34 | 1.33 | | | | |
| | | May 26, 1924 | 0.082 | 0.018 | 0.064 | " | | | | 6.2 | 3.3 | 1.2 |
| G. F. | Chronic nephrosis. | Nov. 27, 1923 | 0.113 | 0.020 | 0.093 | Fermentation. | | 2.70 | 20.6 | 6.7 | 3.3 | 1.4 |
| R. S. | Chronic glomerular nephritis. Stage iii. | Feb. 17, 1925 | 0.117 | 0.070 | 0.047 | Glycolysis. | 7.29 | 4.28 | 103 | 82.3 | 5.3 | 9.1 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | iii. | | | | | | | | | | | |
| E. L. | Chronic glomerular nephritis, vascular type. Stage iii. | Dec. 3, 1923 | 0.137 | 0.010 | 0.127 | Fermentation. | 7.34 | 2.22 | 74 | 47.5 | 4.8 | 4.1 |
| J. L. | Chronic glomerular nephritis, vascular type. Stage iii. | Nov. 26, 1923 | 0.229 | 0.032 | 0.197 | Fermentation. | 7.16 | 3.35 | 207 | 147 | 6.0 | 18.4 |
| | | " 30, 1923 | 0.120 | | | | 7.33 | 4.00 | 214 | 154 | 6.0 | 18.1 |
| | | Dec. 13, 1923 | 0.169 | | | | 7.18 | 3.26 | 162 | 139 | 7.2 | 17.9 |
| | | Jan. 9, 1924 | 0.187 | | | | 7.29 | 3.87 | 182 | 146 | 9.0 | 17.9 |
| J. C. | Chronic glomerular nephritis, vascular type. Stage iii. | Nov. 15, 1923 | 0.100 | 0.038 | 0.062 | Fermentation. | | 1.61 | 62 | 42 | 4.5 | 4.9 |
| | | Apr. 11, 1924 | 0.159 | 0.048 | 0.111 | Glycolysis. | 7.31 | 5.73 | 341 | 287 | 21.3 | 19.7 |
| A. S. | Chronic glomerular nephritis, vascular type. Stage iii. | Apr. 14, 1924 | 0.101 | 0.025 | 0.076 | Glycolysis. | 7.35 | 2.15 | 100 | 68.2 | 6.9 | 8.4 |
| | | May 1, 1924 | 0.135 | 0.044 | 0.091 | " | 7.22 | | | 224 | 11.7 | 19.2 |
| | | " 5, 1924 | 0.191 | 0.046 | 0.145 | " | 7.28 | 5.52 | 278 | 235.8 | 12.3 | 20.3 |
| S. | Nephrosclerosis. | Dec. 11, 1923 | 0.108 | 0.024 | 0.084 | Fermentation. | 7.36 | | 36 | 11.3 | 3.9 | 1.6 |
| W. B. | Nephrosclerosis. | Nov. 16, 1923 | 0.101 | 0.020 | 0.081 | Fermentation. | | | | 15.0 | | |
| F. | Nephrosclerosis. | May 27, 1924 | 0.097 | 0.010 | 0.087 | Glycolysis. | | | | 10.2 | 2.7 | 1.4 |

mal and nephritic bloods. Estimations of non-protein nitrogen, urea, uric acid, creatinine, plasma pH, and serum inorganic phosphorus are also reported in order to afford comparison of other chemical abnormalities in the blood of the nephritic patients with the residual reduction figures.

In the bloods of the normal men, dogs, and horse, the total reducing substance calculated as glucose was from 0.08 to 0.11 per cent, and the residual reducing substance from 0.01 to 0.035 per cent, leaving 0.06 to 0.09 per cent of actual glucose calculated by difference.

The bloods from the two cases of nephrosis[1] and the three of nephrosclerosis gave reducing values within the above normal ranges. None of these cases showed significant nitrogen retention. Of the five cases of chronic glomerulonephritis with nitrogen retention four showed in one or more blood samples abnormally high amounts of total reducing substance, the values ranging up to 0.23 per cent. The non-glycolyzable residual reducing substances were increased to about the slight extent that might be expected from the retained uric acid and creatinine, most of the total reduction values being from 0.02 to 0.05 per cent. In all cases where the total reduction was equivalent to over 0.12 per cent of glucose, the important part of the increase above normal was due to material, presumably glucose, that was destroyed under the conditions of glycolysis.

Marked lowering of the blood plasma pH occurred repeatedly in this group. Its coincidence in several cases with hyperglycemia suggested that the latter might be due to accelerated glycogenolysis caused by the reaction change, since Langfeldt (37) found that the optimum for diastatic activity was much below normal blood pH. However, the correlation between changes in pH and reducing power was by no means constant.

Inorganic phosphorus was also found increased in this group. Despite the demonstrated physiological relationships between phosphate and glucose mobilizations (38), however, no parallelism could be established in our cases.

SUMMARY.

The behavior of the reducing substances in the blood has been

[1] The terms used to indicate the different types of nephritis are those of Volhard and Fahr, discussed in a former paper (36).

studied by subjecting the latter to yeast fermentation and to incubation at 38° under conditions that lead to complete destruction of glucose.

Such treatment leaves in normal blood a residue of reducing substance, other than glucose, which shows a reducing power for ferricyanide (Hagedorn-Jensen method) and for copper (Folin-Wu method) equivalent to 0.01 to 0.03 per cent of glucose.

By inducing insulin shock we succeeded in two out of six rabbits in reducing the fermentable sugar to zero, without affecting the non-fermentable reducing substance.

Abnormally high total reducing power was observed in the blood of four out of five cases of glomerulonephritis with high nitrogen retention. The substance causing the increased reducing power showed, when the blood was fermented or incubated, a behavior identical with that of glucose.

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# HYPERGLYCEMIA PRODUCED BY GLYCEROL.

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We have recently reported observations indicating that glycerol can prevent and relieve the symptoms associated with the hypoglycemia in albino rats following the injection of a minimum lethal dose of insulin (1). The curative effect of an intraperitoneal injection of a 20 per cent glycerol solution in this condition is so prompt, requiring only about 10 minutes, that it approximates the effect produced by the injection of a similar amount of glucose. In rabbits the symptoms can also be relieved, but in this species it is advisable to use larger doses of glycerol (8 to 12 gm. per kilo) in 20 per cent solution *per os*. This antagonistic action of glycerol on insulin may be due primarily to a conversion of glycerol into glucose. In order to test this possibility it was decided to determine first of all the effect of glycerol on the blood sugar concentration of fasted rabbits. So far as we are aware, this question has not been studied by previous workers.

## EXPERIMENTAL.

Healthy, adult rabbits weighing about 2 kilos were kept on a diet of cabbage and oats. Late in the afternoon of the day preceding the experiment the food was removed from the cage. The following morning, after a period of about 18 hours fasting, blood samples were withdrawn from an ear vein. The animals then received a solution of chemically pure glycerol in varying concentration by means of a stomach tube or intraperitoneally. Great care was exercised in the handling of the animals in order to avoid excitement with its possible disturbing action on the blood sugar. Other blood samples were withdrawn at definite intervals for a period of 3 to 5 hours.

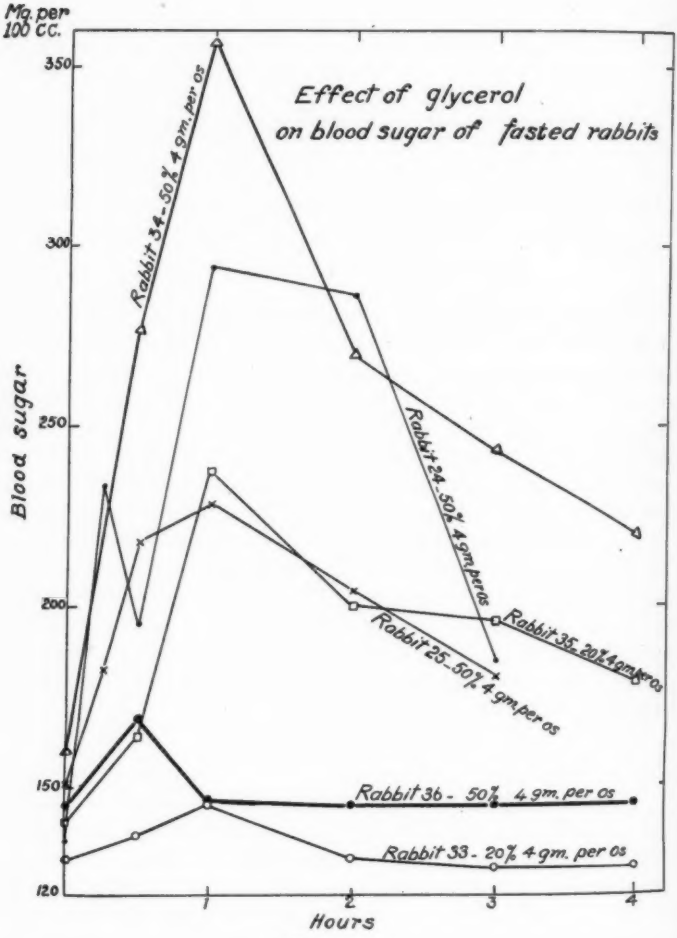


CHART 1.

The blood sugar was determined according to Folin and Wu (2). Control experiments with a dilute glycerol solution indicated that glycerol *per se* does not cause any reduction of the copper test solutions. It appeared, therefore, reasonable to regard any possi-

ble increase of blood sugar following the administration of glycerol as not due to the presence of unchanged glycerol. In order to correlate any change in blood sugar with changes in blood concentration, the total blood solids were determined at each interval.

The results are compiled in Chart 1 and Table I.

TABLE I.

*Influence on Blood Sugar and Blood Solids of 4 Gm. of Glycerol per Kilo of Body Weight in Rabbits, Previously Fasted for 18 Hours.*

| | Blood sugar. | | | | | | | Total solids. | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Hrs. | | | | | | | Hrs. | | | | | | |
| | 0 | ½ | 1 | 2 | 3 | 4 | 5 | 0 | ½ | 1 | 2 | 3 | 4 | 5 |
| | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| Rabbit 40. | | | | | | | | | | | | | | |
| 50 per cent glycerol *per os.* | 104 | 217 | 257 | 244 | 204 | 200 | | 16 | 16 | 16.2 | 15.9 | 16 | 16.2 | 16 |
| Rabbit 44. | | | | | | | | | | | | | | |
| 50 per cent glycerol *per os.* | 169 | 192 | 244 | 188 | 166 | 151 | 161 | 15.8 | 16.3 | 15.1 | 14.8 | 13.9 | 14 | 14.2 |
| Rabbit 43. | | | | | | | | | | | | | | |
| 20 per cent glycerol *per os.* | 109 | 208 | 250 | 204 | 188 | 181 | 156 | 12.6 | 14.7 | 14.4 | 14.9 | 14.1 | 13.7 | 12.7 |
| Rabbit 46. | | | | | | | | | | | | | | |
| 50 per cent glycerol intraperitoneally. | 133 | 196 | 270 | 257 | 232 | 192 | 172 | 17.2 | 18.8 | 18.8 | 17.8 | 17.7 | 17.8 | 18 |
| Rabbit 45. | | | | | | | | | | | | | | |
| 20 per cent glycerol intraperitoneally. | 140 | 196 | 313 | 257 | 244 | 217 | 200 | 15.5 | 16.7 | 16.6 | 16.3 | 15.4 | 14.7 | 14.1 |

DISCUSSION.

It will be noted that in every one of the eleven animals there occurred a rise in blood sugar. The maximum increase is obtained about an hour after the administration of the glycerol and amounts to about 100 per cent. Only two of the rabbits show a relatively

small increase. It is very likely that this may be due to deficient absorption, as the amount of glycerol used was relatively large. The decline in the blood sugar curve after the 1st hour is rather rapid, though some of the values at the end of the 4th hour (Rabbits 40, 43, 46, 45, 34, and 35) are still very high. That this rise in blood sugar cannot be attributed to a concentration of the blood is evident from the figures of the blood solids in the table. It will be noted that there is no corresponding increase in solids.

There is a remote possibility that the increase in blood sugar observed in these experiments is not due to the presence of extra sugar, but rather to the presence of another reducing substance (glyceric aldehyde) derived from glycerol and produced from the latter within the body. If this were true, it would invalidate more or less all the work done with the present methods of blood sugar analysis. On the other hand, we have shown that glycerol has an antagonistic action on the symptoms associated with the hypoglycemia following the injection of insulin and for the present this action of glycerol is best explained by the assumption of an actual conversion into glucose. The work of Cremer (3), Lüthje (4), and Chambers and Deuel (5) on phlorhizin and pancreatic diabetes in dogs indicates that the ingestion of glycerol is followed by the elimination of an amount of extra glucose in the urine approximating the amount calculated on the basis of a complete conversion of glycerol into glucose. Hence there is good reason to consider the hyperglycemia produced by glycerol as due to an actual increase in blood sugar.

SUMMARY.

Glycerol given to fasted rabbits produces a hyperglycemia of considerable extent and duration, indicating a conversion of glycerol into glucose.

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# FERMENTATION PRODUCTS OF CERTAIN MANNITOL-FORMING BACTERIA.*

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The formation of mannitol in fermenting mixtures of plant origin is now known to be due to a group of bacteria which act upon fructose and reduce this sugar to its corresponding alcohol. Mannitol-forming bacteria have been found in wine, sauerkraut, silage, canned goods, yeast, and cereal infusions. In some of these products such as sauerkraut and silage, their presence is not objectionable, for it is probable that they are among the chief microorganisms concerned in the natural souring and preservation of these materials. On the other hand, their presence and activities in the manufacture of wine, canned goods, yeast, and butyl alcohol are the cause of much trouble and financial loss.

It is in relation to the wine industry that this group of bacteria has received special study. The papers of Gayon and Dubourg (1), Müller-Thurgau and Osterwalder (2), and others (3–8) furnish abundant evidence to prove the injurious nature of these microorganisms. The sugars are fermented to mannitol, acetic and lactic acids, and other products which result in a sour wine of lowered alcoholic content. Fruit juices are, however, not the only source of these bacteria. Their presence on cabbage and corn as indicated by the formation of mannitol in sauerkraut and silage has been shown by Feder (9), Nelson and Beck (10), Brunkow, Fred, and Peterson (11), and Dox and Plaisance (12). From these same sources mannitol-producing bacteria have been isolated by Plaisance and Hammer (13), and Fred, Peterson, and Anderson (14). Smit (15), Van Steenberge (16), and Fred, Peter-

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son, and Davenport (17) have obtained them also from yeast, soil, and manure.

The products formed by these microorganisms from the principal sugars have been determined by Gayon and Dubourg (1), Smit (15), and more recently by Fred, Peterson, and their associates (17–19). Pentoses, if fermented, are converted into acetic and lactic acids. Glucose and the other aldoses yield chiefly ethyl alcohol, lactic acid, and carbon dioxide. Besides mannitol, acetic acid, lactic acid, and carbon dioxide are formed from fructose. Sucrose and raffinose are destroyed by most of these bacteria with the production of varying quantities of mannitol. In the fermentation of fructose, mannitol accounts for 50 per cent or more of the sugar. Ethyl alcohol and carbon dioxide are each equivalent to about 25 per cent of the fermented glucose. The formation of mannitol, ethyl alcohol, and carbon dioxide is the most distinctive characteristic of this group of bacteria.

## EXPERIMENTAL.

The mannitol cultures were secured by plating various samples of fermenting cereal infusions. A large number of cultures were secured in this way and their fermentation characteristics studied.

From forty pure cultures thus obtained four were selected for detailed study. These four cultures were replated three times on 0.25 per cent glucose-yeast water agar. Difference in the fermentation of the pentoses formed the basis of this selection. Culture 26 ferments both arabinose and xylose; Culture 19 attacks arabinose only; Culture 36 destroys xylose, but not arabinose; and Culture 23 ferments neither of these pentoses. Many of these organisms are similar to those previously described in the literature. Culture 26 presents a close analogy to *Bacterium mannitopoeum* described by Müller-Thurgau and Osterwalder. Culture 36 is very similar to *Bacillus gayoni* studied by the same investigators. Culture 23 resembles in many respects *Lactobacillus fermentum* described by Beijerinck (20). Except in the fermentation of sucrose, Culture 19 agrees very well in fermentation characteristics with Culture 11 of the beta bacterium described by Orla-Jensen (21).

*Fermentation Characteristics.*

Test-tube fermentations of various sugars and other carbon compounds are not only an aid in classifying organisms, but are also useful indexes as to the type of fermentation. Many sources of carbon were tried in studying the fermentative ability of these organisms. Very little or no acid production was shown with man-

TABLE I.

*Fermentation of Various Sugars.*

Computed for 100 cc. of culture; age, 120 hours.

| Sugar. | Culture 26. | | Culture 19. | | Culture 36. | | Culture 23. | |
|---|---|---|---|---|---|---|---|---|
| | 0.1 N acid. | Sugar fermented. | 0.1 N acid. | Sugar fermented. | 0.1 N acid. | Sugar fermented. | 0.1 N acid. | Sugar fermented. |
| | *cc.* | *per cent* | *cc.* | *per cent* | *cc.* | *per cent* | *cc.* | *per cent* |
| Arabinose | 36 | 22 | 45 | 36 | 2 | 0 | 2 | 0 |
| Xylose | 22 | 15 | 3 | 5 | 35 | 25 | 3 | 3 |
| Rhamnose | 2 | 3 | 2 | 0 | 3 | 5 | 2 | 0 |
| Glucose | 40 | 80 | 36 | 69 | 46 | 84 | 45 | 85 |
| Fructose | 32 | 90 | 35 | 91 | 36 | 91 | 35 | 91 |
| Galactose | 24 | 53 | 36 | 72 | 42 | 84 | 38 | 75 |
| Mannose | 18 | 20 | 44 | 93 | 27 | 33 | 37 | 69 |
| Sucrose | 40 | 79 | 40 | 73 | 54 | 94 | 55 | 97 |
| Maltose | 41 | 81 | 22 | 36 | 45 | 83 | 37 | 74 |
| Lactose | 5 | 16 | 33 | 73 | 28 | 55 | 39 | 80 |
| Melibiose | 40 | 88 | 42 | 83 | 46 | 93 | 45 | 88 |
| Trehalose | 2 | 6 | 1 | 2 | 2 | 3 | 1 | 5 |
| Raffinose | 43 | 78 | 38 | 57 | 42 | 78 | 52 | 77 |
| Melezitose | 2 | | 1 | 2 | 2 | | 1 | 0 |

nitol, dulcitol, dextrin, amygdalin, $\alpha$-methylglucoside, esculin, and salicin. Table I presents data for the fermentation of 100 cc. portions of approximately 1 per cent sugar solutions. By comparing the data given for Culture 19 on arabinose, glucose, and fructose, it is evident that from the latter two sugars neutral products must be formed. Comparing glucose and fructose alone for the same organism it is apparent that more of a neutral

product is formed from fructose than from glucose. These indications are confirmed in the quantitative data which were later obtained.

*Fermentation Products from Various Sugars.*

The analytical methods followed were similar to those published in earlier work (17, 18). The amount of sugar at the beginning and end of the reaction was accurately determined. The major products were isolated and an attempt was made to account for the loss of sugar in a sum of the products.

*Fermentation of Pentoses.*—Approximately 2 per cent solutions of the carbohydrate in yeast water were used in this study. The data are given in Table II and are comparable to those cited in

TABLE II.

*Fermentation Products of Pentose Sugars.*

| Culture No. | Age. | Sugar. | | Acids. | | Carbon dioxide. | Total products. |
|---|---|---|---|---|---|---|---|
| | | Kind. | Amount fermented. | Volatile as acetic. | Non-volatile as lactic. | | |
| | *days* | | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* |
| 26 | 32 | Xylose. | 1.774 | 0.778 | 0.999 | 0.044 | 1.821 |
| 26 | 32 | Arabinose. | 1.268 | 0.558 | 0.686 | 0.032 | 1.276 |
| 26 | 39 | Xylose. | 1.779 | 0.783 | 0.952 | | 1.735 |
| 26 | 39 | Arabinose. | 1.770 | 0.768 | 0.910 | | 1.678 |
| 19 | 20 | " | 1.765 | 0.760 | 0.852 | | 1.612 |
| 36 | 32 | Xylose. | 1.773 | 0.830 | 0.960 | 0.039 | 1.829 |
| 36 | 32 | Arabinose. | 0.079 | 0.012 | 0.0 | 0.014 | 0.026 |
| 36 | 39 | Xylose. | 1.774 | 0.752 | 0.854 | | 1.606 |
| 36 | 39 | Arabinose. | 0.105 | 0.008 | 0.006 | | 0.014 |

previous publications. If attacked at all, the pentose molecule is cleaved into lactic and acetic acids. These are the major products of the fermentation. Practically no alcohol is formed, and carbon dioxide is produced only in traces. Recovery of products ranges from 90 to 103 per cent. However, the ratio of acetic to lactic acid does not correspond with the theory of 1:1.5 for a simple cleavage. It is observed to vary from 1:1.12 to 1:1.28, which indicates a side reaction of some nature. As no appreciable quantities of carbon dioxide appear, there cannot be a secondary fer-

mentation of lactic acid. A possible explanation may lie in an aldol condensation of intermediate aldehydic products and their subsequent conversion to acetic acid. Thus 2 molecules of an aldehyde with 3 carbon atoms in its molecule might unite to form an intermediate compound with a chain of 6 carbon atoms which could be fermented to acetic acid.

Several of the cultures were set up with an excess of sterile $CaCO_3$ present. As the major fermentation products are acids, this method is to be preferred to that of successive alkali titrations. Although not adapted for a carbon dioxide determination, yet because of the constancy of its favorable hydrogen ion concentration, this method is conducive to a rapid and complete fermentation.

Some of these organisms are limited in their choice of pentose sugars. In the results of Table I Culture 36 is shown to attack xylose vigorously and to leave arabinose practically untouched. This is equally true with large quantities of sugar. Even in the presence of $CaCO_3$ the culture containing arabinose showed but very little destruction of sugar. The organism apparently uses just enough of the carbohydrate to keep itself alive with slow reproduction. Culture 36 shows marked differences between the pentoses which can be accounted for only by the different space arrangements of the sugar molecule. As will be shown later, xylose is as completely destroyed as glucose which is usually considered the most readily fermented sugar. The similarity of structure of these two sugars may be offered as an explanation for the practically complete destruction of xylose. Culture 19 distinguishes between these two pentoses in an opposite manner. It shows a strong preference for arabinose and scarcely consumes xylose at all. Because of the corresponding similarity in structure between arabinose and galactose, this organism should be a poor fermenter of glucose and a strong fermenter of galactose and lactose. The figures of Table I in general bear out this relationship. Culture 26 destroys both of the pentoses, and Culture 23 attacks neither of these sugars. Rhamnose is not a favorable sugar for any of these organisms.

*Fermentation of Fructose and Fructo Sugars.*[1]—The initial quali-

[1] Fructose-yielding sugars.

tative fermentation of fructose offered an interesting possibility. The sugar was rapidly and almost completey destroyed with only a moderate production of acid. In the larger fermentations all of the products were not accounted for in the regular scheme of analysis. A major reaction product, which was found to be mannitol, was still in the residue of the lactic acid extraction. The quantity of this hexahydric alcohol was determined by evaporating the residue from the ether extraction to dryness in a porcelain

TABLE III.

*Fermentation Products of Fructose, Sucrose, and Raffinose.*

| Culture No. | Age. | Sugar. | | Acids. | | Alcohol as ethyl. | Carbon dioxide. | Mannitol. | Total products. |
|---|---|---|---|---|---|---|---|---|---|
| | | Kind. | Amount fermented. | Volatile as acetic. | Non-volatile as lactic. | | | | |
| | *days* | | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* |
| 26 | 14 | Fructose. | 1.626 | 0.217 | 0.300 | 0.0 | 0.142 | 0.823 | 1.482 |
| 19 | 9 | " | 1.580 | 0.201 | 0.308 | 0.0 | 0.150 | 0.780 | 1.439 |
| 36 | 12 | " | 1.667 | 0.235 | 0.202 | 0.0 | 0.102 | 0.965 | 1.504 |
| 23 | 9 | " | 1.578 | 0.215 | 0.268 | 0.0 | 0.133 | 0.774 | 1.390 |
| 26 | 16 | Sucrose. | 1.817 | 0.087 | 0.756 | 0.401 | 0.403 | Trace. | 1.647 |
| 19 | 15 | " | 1.842 | 0.081 | 0.717 | 0.372 | 0.354 | 0.119 | 1.643 |
| 36 | 15 | " | 1.857 | 0.074 | 0.614 | 0.374 | 0.386 | 0.161 | 1.709 |
| 23 | 20 | " | 1.857 | 0.091 | 0.707 | 0.365 | 0.394 | 0.074 | 1.631 |
| 41–11 | 22 | " | 1.499 | 0.127 | 0.566 | 0.260 | | 0.0 | 0.953 |
| 26 | 16 | Raffinose. | 1.630 | 0.103 | 0.576 | 0.382 | 0.237 | +* | 1.298 |
| 36 | 16 | " | 1.435 | 0.085 | 0.493 | 0.287 | 0.302 | +* | 1.167 |

* Definite crystals, but sugar residue interfered with quantitative separation.

dish containing a layer of washed sand, and then extracting the mannitol with hot 80 per cent alcohol. The solution was evaporated and the mannitol crystallized in three fractions at a low temperature. However, all of the mannitol cannot be separated in this way, and for this reason the sum of the products in Table III is somewhat low.

The nature of mannitol formation from fructose has been previously described. The first fermentation products are probably lactic and acetic acids, carbon dioxide, and hydrogen. This

reducing agent then converts half or more of the fructose to mannitol, which is not readily susceptible to a secondary fermentation.

The fructo sugars, sucrose and raffinose, are readily fermented by these organisms. Sucrose, however, is quite readily hydrolyzed by heat and precautions must be taken in its sterilization. The sugar used for these fermentations was sterilized in carbon dioxide-free water in 12 per cent solution at 15 pounds pressure for 20 minutes. The reducing sugar content after this treatment was found not to exceed 8 mg. per gm. of sucrose. The fact that Culture 26 yielded but a mere trace of mannitol is additional evidence that no appreciable quantities of invert sugar were present at the time of inoculation.

From the figures of Table III it is evident that the sucrose molecule is not first hydrolyzed to glucose and fructose and these hexoses then fermented. If hydrolysis occurred, there would be produced, as compared with equivalent quantities of glucose and fructose, about half as much mannitol as is obtained from fructose, a decreased amount of alcohol, and an increased percentage of volatile acid and carbon dioxide. But this is not the case, and the sucrose molecule must be either fermented directly or, if hydrolyzed, the fructose component must be in some enolic variation which is more susceptible to an alcohol than to a mannitol fermentation.

*Lactobacillus pentoaceticus* was included in the sucrose fermentations, but did not attack the sugar until $CaCO_3$ was added. Here again the more favorable hydrogen ion concentration of a medium containing $CaCO_3$ is evidenced. Even after the addition of this neutralizing agent the fermentation was not complete. No mannitol was found. The increased volatile acid content is probably due to a secondary fermentation of lactic acid, for, as previously reported, this organism ferments lactates.

Raffinose is fermented fairly rapidly, but not all of the sugar is consumed. There are two possibilities in the hydrolysis of this trisaccharide. Either sucrose and galactose or melibiose and fructose may be the first hydrolytic products. It it were hydrolyzed in the latter way, mannitol formation would likely follow; if hydrolyzed in the former way, mannitol could be formed only from the sucrose and would be formed only by those organisms which ferment sucrose with the production of mannitol. As

Culture 26 does not form mannitol from sucrose but does produce it from raffinose, the hydrolysis of raffinose to melibiose and fructose is more probable. Culture 36 can produce mannitol from either sucrose or fructose, and either type of hydrolysis might take place.

*Fermentation of Other Sugars.*—As shown in Table IV the fermentation of glucose, galactose, and lactose is marked by the production of alcohol. Lactic acid and carbon dioxide are the other major products. Volatile acid is a minor product. Lac-

TABLE IV.

*Fermentation Products of Aldoses, Calcium Lactate, and Mannitol.*

| Culture No. | Age. | Carbon compound. | | Acids. | | Alcohol as ethyl. | Carbon dioxide. | Total products. |
|---|---|---|---|---|---|---|---|---|
| | | Kind. | Amount fermented. | Volatile as acetic. | Non-volatile as lactic. | | | |
| | *days* | | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* | *gm. per 100 cc.* |
| 26 | 9 | Glucose. | 1.842 | 0.061 | 0.942 | 0.424 | 0.441 | 1.868 |
| 19 | 16 | " | 1.715 | 0.087 | 0.729 | 0.408 | 0.435 | 1.659 |
| 36 | 9 | " | 1.889 | 0.055 | 0.958 | 0.442 | 0.439 | 1.894 |
| 23 | 16 | " | 1.813 | 0.072 | 0.779 | 0.431 | 0.429 | 1.711 |
| 26 | 22 | Galactose. | 0.835 | 0.009 | 0.437 | 0.182 | 0.182 | 0.810 |
| 36 | 21 | " | 1.547 | 0.024 | 0.645 | 0.410 | 0.404 | 1.483 |
| 26 | 20 | Lactose. | 0.066 | 0.0 | 0.0 | 0.010 | | 0.010 |
| 36 | 21 | " | 1.525 | 0.033 | 0.721 | 0.452 | 0.466 | 1.672 |
| 36 | 114 | Calcium lactate. | 0.100 | 0.076 | 1.100* | | | 0.076 |
| 36 | 118 | Mannitol. | | 0.220 | 0.332* | 0.0 | | 0.552 |

* Control contained 1.200 gm.

tose is a differentiating carbohydrate for Cultures 26 and 36. The former does not attack it, whereas the latter destroys 90 per cent of the sugar. Culture 36 also shows a more vigorous destruction of galactose than does Culture 26. The type of cleavage is given by glucose, and the other two aldose sugars conform very closely to it. Lactose is probably hydrolyzed to its component hexoses which are then fermented.

*Destruction of Products.*—The possibility of secondary fermentations is also given by the figures of Table IV. Culture 36 very slowly destroys calcium lactate as shown by the production of

volatile acid. Also, it is the only organism of this group which ferments mannitol. A 2 per cent solution of the polyhydric alcohol was used. Destruction of this fermentation product results in the production of lactic and acetic acids, and presumably of

TABLE V.

*Identification of Products.*

| Culture No. | Carbon compound. | Ba content of volatile acid. | Ba content of acid from alcohol. | Water of crystallization in first crop of Zn lactate. |
|---|---|---|---|---|
| | | *per cent* | *per cent* | *per cent* |
| 26 | Xylose. | 53.1 | | 18.12 |
| 36 | " | 53.1 | | 18.15 |
| 26 | Arabinose. | 52.9 | | 18.11 |
| 19 | " | 53.2 | | 18.16 |
| 26 | Glucose. | 50.1 | 53.3 | 17.98 |
| 19 | " | 51.4 | 53.7 | 18.14 |
| 36 | " | 50.1 | 53.9 | 18.16 |
| 23 | " | 52.3 | 53.3 | 18.16 |
| 26 | Fructose. | 53.8 | | 18.00 |
| 19 | " | 53.7 | | 18.17 |
| 36 | " | 53.0 | | 18.23 |
| 23 | " | 53.0 | | 18.11 |
| 26 | Galactose. | | 53.4 | 18.28 |
| 36 | " | 54.1 | 53.3 | 18.07 |
| 36 | Lactose. | 51.1 | 53.7 | 18.13 |
| 26 | Sucrose. | 50.8 | 53.7 | 18.14 |
| 19 | " | 51.2 | 53.4 | 18.13 |
| 36 | " | 50.6 | 53.5 | 18.15 |
| 23 | " | 50.9 | 53.6 | 18.14 |
| 41–11 | " | 52.0 | 52.9 | 18.11 |
| 26 | Raffinose. | 50.9 | 53.2 | 18.20 |
| 36 | " | 53.8 | 53.2 | 18.19 |
| 36 | Mannitol. | 52.8 | | 18.25 |
| Theory for Ba in $Ba(C_2H_3O_2)_2$ | | 53.8 | 53.8 | |
| Theory for $H_2O$ in $Zn(C_2H_3O_2)_2 + 3H_2O$ | | | | 18.17 |

carbon dioxide. This fermentation proceeds with extreme slowness, for in 118 days only a little more than 25 per cent of the mannitol was destroyed.

*Identification of Volatile Acid and Alcohol.*—Volatile acid is readily identified by making a clear solution of the barium salt,

evaporating a desired aliquot to dryness in a platinum dish, drying to constant weight at 130°C., weighing, converting the barium present to the sulfate, and computing the barium content of the original salt. Alcohol was, in the course of its determination, converted to the corresponding volatile acid which was then identified. The results are given in Table V. The majority of the volatile acid samples are low in barium content. This is no doubt due to the small amount of lactic acid which passes over with the volatile acid, the barium content of which is 43.5 per cent. The amount would be influenced by the proportions of each acid present and the length of the distillation.

*Form of Lactic Acid Produced.*—The form of lactic acid produced is conveniently identified by the water of crystallization of its zinc salt. The physical appearance of the crystallizing lactate generally indicates which form of salt is present. Inactive zinc lactate comes out of solution very readily and is quite flaky. Active zinc lactate is much more soluble, crystallizes from small volumes of the mother liquor, and the particles, which are very fine, form a cake on the filter. Table V also gives the data for these salts. Most of the lactic acid produced from all of these organisms is inactive, and the results are in nearly every case in close agreement with the theoretical.

*Description of Organisms.*—All of these organisms are Gram-positive rod forms, non-motile, non-spore formers, catalase-negative, do not liquefy gelatin, grow best in an atmosphere of lowered oxygen tension, and have a thermal death-point in a neutral medium between 60° and 70°C. Cultures 26, 23, and 36 are small rods 0.4 to 0.6 by 1.2 to 2.8 microns. The cells of Culture 19 are much larger, 0.6 to 0.75 by 2.0 to 9.0 microns, and occur singly or in chains. Carbolfuchsin is perhaps the best stain, but thionin is almost equally satisfactory. Culture 26 does not curdle milk; the others produce acid and form curd in from 10 to 20 days. Floccule formation in the fermented medium is evidenced by Cultures 26 and 19 only. Methylene blue is reduced by all the cultures but more rapidly by Culture 36. The stronger reducing power of this organism is also shown by its being the only one which reduces nitrates, and again by the better yields of mannitol obtained from both fructose and sucrose.

SUMMARY.

Four strains of mannitol-forming bacteria have been isolated from cereals and their fermentation characteristics and products determined.

Culture 26 ferments both xylose and arabinose; Culture 19 destroys only arabinose; Culture 36 attacks xylose only; and Culture 23 ferments neither of these sugars. The pentose molecule is cleaved with the production of lactic and acetic acids.

Fructose is completely fermented with the formation of lactic and acetic acids, carbon dioxide, and mannitol. All of the cultures ferment sucrose and three of them produce small quantities of mannitol from it. Raffinose is partially destroyed by Cultures 26 and 36 with the production of lactic and acetic acids, alcohol, carbon dioxide, and mannitol.

Glucose is fermented with the production of ethyl alcohol, carbon dioxide, and lactic acid. Galactose and lactose are attacked to a lesser degree, but if fermented, result in the formation of similar products.

Irrespective of the sugar fermented, the lactic acid produced was found to be mainly inactive. Calcium lactate is slowly fermented by Culture 36 with the production of volatile acid. The same organism slowly destroys mannitol with the formation of lactic and acetic acids, and probably of carbon dioxide.

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# PROTEINS OF THE BARK OF THE COMMON LOCUST TREE, ROBINIA PSEUDACACIA.

## I. ENZYMES ASSOCIATED WITH THE PROTEINS: THE COMPOSITION, PROPERTIES, NITROGEN DISTRIBUTION, AND SOME OF THE AMINO ACIDS OF THE ALBUMIN.*

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The first recorded instance, as far as we are aware, in which protein has been shown to occur in the bark of a tree is that noted by Power and Cambier (1, 2), who isolated from the bark of the common locust tree protein having toxic properties. They found that when an aqueous extract of the light colored, inner bark was heated, a flocculent coagulum was produced, the largest quantity separating between 70° and 80°C. The protein was also precipitated from its aqueous solution by addition of several volumes of strong alcohol. The yield of dry substance thus obtained ranged from 1.1 to 1.7 per cent of the weight of the air-dried bark used. This bark contained 2.82 per cent of nitrogen. They described the *Robinia* protein as being soluble in water and precipitable from its solutions by mineral acids. It responded positively to the usual tests and reactions which are characteristic of proteins. It was observed that a saline solution extracted a larger quantity of protein from the bark than did water alone, and that when the extract was heated the protein did not coagulate at a uniform temperature. They, therefore, concluded that the bark probably contained more than one protein. The protein precipitate obtained by adding alcohol to an aqueous extract of the bark was shown to have enzymic properties. It effected the hydrolysis of both amygdalin and sinigrin, with the formation respectively of bitter almond oil (benzaldehyde and hydrocyanic acid) and mustard oil, and, like the rennet ferment, coagulated the casein of milk. Heating the protein destroyed the enzymic properties. Power and Cambier found also that the protein material precipitated by alcohol possessed marked toxic properties. When given internally to a dog

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in a dose corresponding to 30 gm. of the bark, a severe attack of vomiting ensued within 15 minutes and continued at intervals for about 6 hours. A similar quantity of the protein which had been heated to 100°C. had no toxic effect. An aqueous extract, representing 5 gm. of the bark, taken by one of the authors cited, caused violent vomiting and purging.

The name *robin*[1] was subsequently given by Lau (3) to designate collectively the proteins of the *Robinia* bark which were obtained as described by Power, by precipitation with alcohol or ammonium sulfate. Lau showed that hypodermic injections of a solution of robin caused an alteration of the kidneys of rabbits, often producing a nephritis, manifested by the presence of albumin, hemoglobin, and cylindrical casts in the urine.

Later Kobert (4) found that solutions of robin, even in great dilutions, agglutinated the red corpuscles of the blood of many different kinds of animals. Unlike ricin, however, robin did not agglutinate the red corpuscles of dog's blood.

Ehrlich (5) showed that when robin is injected into animals there is produced an antitoxin corresponding in its properties to antiricin, although the two initial proteins, robin and ricin, are entirely different.

Numerous instances are recorded where both people and animals have been poisoned by the bark of the locust tree.

Power and Cambier (1) refer to a case where horses were made violently sick by swallowing some of the locust bark.

Emery (6) has reported that thirty-two boys were poisoned by chewing the inner bark of this tree, and describes the symptoms as follows: "In the severest cases large quantities of ropy mucus mixed with blood were vomited: the other symptoms were retching, pain in the epigastrium, debility, stupor, extremities cold and pulseless, heart's action feeble and intermittent, pupils dilated, faces of a dusky pallor."

Lewin (7) gives an account of cattle becoming violently sick within an hour after chewing the bark.

Wolberg (8) cites an instance where seven horses became sick as a result of eating *Robinia* bark and three died.

Kobert (4) refers to the death in France of six army horses and three cows from having eaten the bark. Several other instances where animals have suffered from the toxic effect of the *Robinia* bark are recorded.

Regarding the physiological properties of the other parts of the locust tree not much information seems to have been recorded. Power and Cambier (1) have referred to an instance where three children were poisoned

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[1] Inasmuch as the writers have found that more than one protein exist in the bark of the locust tree, it will be desirable to limit the use of the name *robin* to only one of the proteins. It would seem most appropriate to apply this name to that protein which is responsible for the toxic properties that have been ascribed to the *Robinia* proteins in general. However, until we have more information regarding the physiological properties of the individual proteins, the use of the name robin will be retained to designate the *Robinia* proteins collectively.

by eating the root of the *Robinia*. The symptoms were similar to those produced by an overdose of belladonna. They also cite a case where a child who had eaten of the seeds became sick and manifested symptoms like those produced by the bark.

Holmes (9) has directed attention to the poisonous properties of the pods of *Robinia pseudacacia*, and cites a recent instance of the fatal poisoning of ewes through eating the dry pods.

The leaves of the locust tree appear to be devoid of poisonous properties (10) inasmuch as they were recommended many years ago as affording wholesome food for cattle. Several references relating to this use of the leaves, one of which dates from the year 1786, have been recorded by Power and Cambier (1).

In a recent publication Kern (11) gives an account of the presence of numerous cells, filled with highly refractive microscopic round bodies, in the bark parenchyma of several varieties of trees. The behavior of this substance toward heat, saturated salt solutions, strong alcohol, and the various reagents used for the characterization of proteins indicated the presence of a labile form of protein. These observations were made when working with bark collected during the winter. Bark collected in the summer, however, contained but little of this refractive substance in the parenchyma cells. These results are correlated with those of the investigations of Schulze (12), who showed that soon before the leaves fall in the autumn their protein is transported to the parenchyma of the bark, this transference involving, first, a cleavage of the protein, yielding amino acids, from which protein is again regenerated and stored in the bark.

In view of the twofold interest connected with the protein of the locust bark—its toxic properties and the fact that it opens up a new and wide field for protein investigation in a domain to which but little attention has been hitherto paid—we have undertaken an investigation of its proteins and other nitrogenous constituents.

Working with the air-dried inner bark of the locust tree, containing 2.8 per cent of nitrogen (calculated on a moisture-free basis), we have been able to isolate 2.52 per cent of an albumin and 1.38 per cent of a globulin. A significant quantity of a substance having the properties of proteose was also obtained. Evidence was obtained showing the presence in very small quantity of another low coagulating protein.

It was found that 64 per cent of the total nitrogen of the bark could be removed by exhaustive extractions, first with distilled water and then with 10 per cent sodium chloride solution. Subsequent extraction of the residue with alcohol and with dilute alkali removed but a relatively small amount of nitrogen, which was of a non-protein character, indicating the absence of proteins of the

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prolamin or glutelin type in this bark. The results of the extraction experiments are given in Table I.

The proteins of the locust bark are soluble in very dilute saline solutions, and are precipitated almost completely from a 10 per cent sodium chloride solution by making this solution 0.44 saturated with ammonium sulfate. In this connection it is of interest to point out the low degree of saturation with ammonium sulfate at which the locust bark albumin is precipitated from its solution. Addition of ammonium sulfate to an aqueous solution of the albumin until the solution is a little more than 0.4 saturated precipitates practically all of the albumin. It was formerly believed that albumins were not precipitated at such a low concentration

TABLE I.

*Nitrogen Removed from Air-Dried Locust Bark by Exhaustive Extractions with Various Solvents.**

(Weight of the air-dried bark used = 20.165 gm.)

| Solvent. | Nitrogen. | Total nitrogen. | Protein of the bark (N x 6.25). |
|---|---|---|---|
| | *gm.* | *per cent* | *per cent* |
| Distilled water | 0.2850 | 54.69 | 9.57 |
| Sodium chloride (10 per cent) | 0.0489 | 9.36 | 1.64 |
| Alcohol (70 per cent) | 0.0035 | 0.67 | 0.12 |
| Sodium hydroxide (0.5 per cent) | 0.0070 | 1.34 | 0.23 |
| Total | 0.3444 | 66.06 | 11.56 |

* The percentages are expressed in terms of the moisture-free bark. The air-dried bark contained 7.7 per cent of moisture.

of ammonium sulfate, and the statement is still made in some textbooks that when ammonium sulfate is added to one-half saturation albumin is not precipitated from its solutions at ordinary temperatures.

The crude protein obtained by precipitation either with ammonium sulfate or with alcohol was found to have enzymic properties. Not only was the presence of an enzyme capable of hydrolyzing amygdalin demonstrated, thus confirming the results previously reported by Power, but also the presence of an urease. When the protein substance which had been precipitated from a 10 per cent sodium chloride extract of the bark by addition of ammonium sulfate was dissolved in water and the solution

dialyzed, the globulin which separated still retained the enzymic properties of the original ammonium sulfate precipitate. On the other hand, neither the dialysate nor the isolated proteins obtained from the dialysate had appreciable effect on urea or amygdalin. Purification of the globulin, by reprecipitation by dialysis, and by subsequent thorough washing, not only failed to diminish the enzymic properties, but the purified product possessed these properties to an even greater degree. With reference to the isolated proteins, therefore, the enzymic properties are associated with the globulin of the bark and not with the albumin or proteose.

Several investigators (13–16) record work done on "*Robinia* urease." The urease referred to in these publications, however, is that of the seeds of the locust tree, not of the bark.

The present paper gives the results of our analyses of the albumin, showing its composition, distribution of nitrogen, percentages of amino acids, and general properties. Data on the globulin and other nitrogenous constituents of the locust bark will be published later. We have as yet made no study of the toxic properties of the locust bark proteins. It will be of interest to learn to which, if any, of the proteins which we have isolated are to be attributed the toxic properties which have been ascribed to robin. Studies to this end are expected to be taken up as soon as we have completed our projected chemical work with these proteins.

The albumin isolated from the locust tree bark was found to have about the same general properties as those which characterize the numerous albumins which have been isolated from various seeds.

It can be isolated by the methods generally used for the separation of the albumins of seeds—by heating an extract of the bark or a solution of the proteins precipitated by ammonium sulfate, after having first removed the globulin by dialysis. After drying the coagulum with alcohol and ether in the usual way, the albumin is obtained in a form which can readily be reduced to a light, nearly pure-white powder. Almost all of the preparations obtained were relatively low in ash, and were free from phosphorus. Molisch's test and that with orcinol-hydrochloric acid were positive, showing that small quantities of carbohydrates were present.

The closely agreeing results of the elementary analyses of several different preparations of the albumin are given in Table II.

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The distribution of nitrogen in the albumin was determined by the Van Slyke method. The results obtained show that this protein contains relatively high percentages of the basic diamino acids.

TABLE II.

*Average Results of Duplicate Analyses of Different Preparations Showing the Elementary Composition of the Locust Bark Albumin.*

(The results are expressed in percentages of the ash- and moisture-free protein.)

| Albumin preparation. | C | H | N | S | Ash. |
|---|---|---|---|---|---|
| | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 23-A | 54.35 | 6.85 | 14.75 | 0.76 | 0.57 |
| 27-C | 54.75 | 6.74 | 14.66 | 0.89 | 1.14 |
| 25-B | 53.78 | 6.90 | 14.93 | 0.81 | 0.52 |
| 29-D | 54.33 | 6.76 | 14.70 | 0.76 | 0.55 |
| 31-E | 54.84 | 7.00 | 14.87 | 0.76 | 0.53 |
| 33-F | 54.38 | 6.88 | 14.61 | 0.81 | 0.38 |
| 35-G | 54.71 | 6.81 | 15.02 | 0.81 | 0.17 |
| 37-H | 54.86 | 6.69 | 14.55 | 0.82 | 0.25 |
| 39-J | 54.72 | 6.89 | 14.84 | 0.76 | 0.23 |
| Average..... | 54.52 | 6.83 | 14.77 | 0.80 | |

TABLE III.

*Percentages of Some of the Amino Acids in the Locust Bark Albumin.*

| Amino acids. | |
|---|---|
| | *per cent* |
| Cystine | 1.37 |
| " * | 1.03 |
| Arginine | 4.39 |
| Histidine | 1.74 |
| Lysine | 5.45 |
| Tryptophane* | 4.18 |
| Tyrosine* | 6.27 |
| Aspartic acid | 7.72 |
| Glutamic acid | 4.48 |

* Estimated colorimetrically.

Tyrosine and tryptophane were estimated colorimetrically. Attention is called to the unusually high percentages found for these two amino acids. The aspartic and glutamic acids in the protein were separated from the other products of hydrolysis in the form

of their barium salts, and finally isolated and weighed as pure copper aspartate and glutamic acid hydrochloride. The percentages of the different amino acids found are given in Table III.

## EXPERIMENTAL.

*Preparation of the Material.*—The bark used in this investigation was collected about the middle of August from the trunks and the larger branches of two locust trees, having diameters of about 6 and 10 inches when measured 2 or 3 feet from the ground. The outer, brown, suberous portion of the bark was carefully removed and only the inner, light colored portion employed. The latter was allowed to dry by exposure to the air and afterward ground in a mill to a coarse powder. The material thus prepared contained 7.7 per cent of moisture and 2.81 per cent of nitrogen (calculated on a moisture-free basis), which, when multiplied by the conventional factor 6.25, is equivalent to 17.5 per cent of protein. In view of the statements made by Schulze and Kern, to which references have been made already, it is very likely that the protein content of the bark would be found to vary with the age of the trees and with the season of the year. Not all of the nitrogen of the bark, however, is protein nitrogen.

*Coagulation Tests.*—When an aqueous extract of the bark was slowly heated, a very small quantity of a flocculent precipitate formed at about 48°C. The filtrate from this precipitate on reheating remained perfectly clear up to 60° C. At 61–65° a much larger quantity of coagulum separated. On reheating the filtered solution a further coagulation occurred between 76° and 83°. These coagulation tests indicated the presence of more than one protein—a trace of albumin coagulating at about 48°, an albumin occurring in larger quantities which coagulates at 61–65°, and a globulin coagulating between 76° and 83°. It was observed that a larger quantity of coagulum was obtained at 48–50° when an extract of fresh bark was used than when that of the air-dried material was employed. This suggests that the protein which coagulates at a low temperature may have become partly denatured during the drying, on account of the action of enzymes or acids present in the bark.

*Precipitation Tests with Ammonium Sulfate.*—Precipitation tests were made by adding a saturated aqueous solution of ammonium

sulfate to 100 cc. of a 10 per cent sodium chloride extract of the bark. A slight cloudiness followed the addition of the first 5 cc. portion of ammonium sulfate; and after the addition of 20 cc. a relatively large quantity of a flocculent precipitate separated. The solution was then at 0.167 of saturation. Another flocculation took place at about 0.3 of saturation. A somewhat smaller quantity was again precipitated, when the solution was made 0.41 saturated. Further additions of solid ammonium sulfate to complete saturation produced only a slight turbidity with no flocculation. Practically all of the protein in the extract was precipitated at 0.44 of saturation, the largest quantity separating at 0.3 to 0.4 of saturation.

*Exhaustive Extractions of the Bark.*—A quantity of the air-dried bark weighing 20.16 gm. and containing 0.5230 gm. of nitrogen was extracted successively with distilled water, 10 per cent sodium chloride solution, 70 per cent alcohol, and 0.5 per cent sodium hydroxide solution. The extractions with each solvent were continued until practically no nitrogen was removed. During the extractions with distilled water and with sodium chloride solutions, toluene was used as a preservative. From 100 to 200 cc. of solvent were used for each extraction. Five extractions with distilled water were made at room temperature for periods ranging from 4 to 24 hours each. The first extracts were straw-colored, and showed a distinctly acid reaction to litmus. The bark residue was then similarly extracted four times with the salt solution. These extracts were colorless. Three extractions were subsequently made with alcohol by allowing the bark, which remained after the extraction with sodium chloride solution, to stand with the solvent for several hours at room temperature with occasional stirring, and finally by boiling for 30 minutes in every case. The two extractions with alkali at room temperature gave slightly yellow solutions, which filtered slowly. The results of these extractions are summarized in Table I. It is seen that 66.06 per cent of the total nitrogen of the bark was removed, a little over four-fifths of which was extracted by distilled water.

It is possible that a higher percentage of the total nitrogen could be extracted if fresh bark, rather than the air-dried material, were employed, since coagulation tests made on both fresh and air-dried bark indicated that some of the protein becomes denatured when

the bark is dried by long exposure to the air. Examination of both the alcoholic and alkaline extracts showed that but little, if any, of the small amount of nitrogen they contained was protein nitrogen.

Addition of water to the 10 per cent sodium chloride extract of the bark, even to 40 volumes, produced only a faint turbidity. Dilution of the salt extract of the bark cannot therefore be used as a method for the separation of the globulin.

*Enzymes Associated with the Robinia Proteins.*

Qualitative tests were made with both the mixture of crude proteins and also with the isolated proteins to ascertain their enzymic behavior toward urea and amygdalin. These tests were made by adding small quantities of urea and amygdalin to 4 cc. portions of dilute aqueous or saline solutions of the respective protein materials to be examined, and allowing the mixture to stand at room temperature.

Several volumes of 95 per cent alcohol were added to a 10 per cent sodium chloride extract of the bark, and the precipitate was allowed to stand overnight to settle. Most of the supernatant liquid was decanted, and the residue centrifugated. The precipitated proteins had no effect on either urea or amygdalin. Even after standing for 5 days, the solution containing the urea was neutral to litmus, and no odor of benzaldehyde was detected in the tube containing the amygdalin. The experiment was repeated in the same manner, with the exception that after precipitation with alcohol the proteins were removed at once by filtration and dried. When the material was then tested with urea and amygdalin, distinct odors of ammonia and benzaldehyde respectively were detected after standing for about 2 hours. It is evident, therefore, that in the first experiment the prolonged contact with alcohol had destroyed the activity of the enzymes.

The crude proteins precipitated from a sodium chloride extract of the bark by the addition of ammonium sulfate were likewise tested. Within 30 minutes after adding urea to the protein solution a distinct odor of ammonia was noted. The effect of the protein solution on amygdalin was indicated at the end of 5 hours by a faint odor of benzaldehyde, which became very marked after the mixture had stood overnight.

In order to learn whether these enzymic properties were associated more with one of the proteins than with the other, a quantity of the crude protein mixture which had been precipitated with ammonium sulfate was dissolved by the addition of water and the solution filtered, and the clear filtrate dialyzed until practically free from chlorides and sulfates. The globulin which had separated was collected by centrifugation and washed several times with distilled water. A small portion of the globulin thus prepared was then tested as in the preceding experiments. It was found to be fully as effective in decomposing both urea and amygdalin as was the original crude mixture of proteins precipitated by ammonium sulfate. The dialysate, on the other hand, from which the globulin had separated and which had a pH of about 6.7, had practically no effect on the urea solution. Even after standing for 2 days no odor of ammonia was detectable and the solution had a pH of only about 7.4. In the test with amygdalin the odor of benzaldehyde could be detected only after the mixture had stood for 2 days.

The main portion of the globulin was suspended in 10 per cent sodium chloride solution. The greater part of the globulin had become denatured and would not redissolve. This insoluble portion was filtered off, and the dissolved protein again separated by dialyzing for 10 days. Toluene was added as a preservative. The redialyzed globulin was then washed repeatedly, using each time about 200 times its weight of distilled water. Thus prepared, the globulin was entirely soluble in dilute salt solution. Its behavior was then tested toward urea and amygdalin. Addition of a small quantity to a solution of urea caused the solution to become almost at once strongly alkaline, and within 10 minutes a strong odor of ammonia was evolved.

In the test with amygdalin a distinct odor of benzaldehyde was noticed at the end of $1\frac{1}{2}$ hours.

The globulin was thus found to be decidedly more effective in its enzymic properties after having been precipitated the second time by dialysis than it was after the first precipitation. This is probably to be explained by the fact that the greater part of the globulin which had become denatured after the first dialysis had been discarded and that the portion which did dissolve represented the final and more active product, the greater activity of which

was due to the removal of the inert, denatured globulin. This strongly suggests that the enzymic properties described are not functions of the globulin itself, but that they are due to associated enzymes which have certain properties in common with the globulin, and which are not shared by the albumin.

*Preparation of the Proteins.*

For the preparation of the proteins 500 or 1,000 gm. portions of the air-dried ground bark were extracted with 3 or 6 liters of 10 per cent sodium chloride solution by allowing the mixture to stand for 24 hours or longer at room temperature with occasional stirring. The extracts showed an acid reaction when tested with litmus and had a pH of about 5.1. Larger yields of the proteins were obtained when normal aqueous sodium hydroxide was added to the salt solution, before making the extraction, in sufficient quantity to render an extract of the bark nearly neutral but slightly on the acid side. The amount of alkali required was determined in a preliminary test by titrating the extract from a known quantity of the bark. After the extraction, the mixture was placed in a muslin bag and most of the extract squeezed out by hand, and then as much more as possible by means of a small cider press. The residue was extracted a second time in the same manner, using only half as much solvent and without the addition of any sodium hydroxide. The united extracts were filtered through a thin mat of paper pulp which had been previously washed with 10 per cent salt solution. The straw-colored filtrate was perfectly clear. Proceeding from extracts thus obtained, a large number of preparations of albumin and of globulin were made by methods varying more or less in the different cases, of which the following are representative examples.

(1). The salt extracts of 500 gm. of bark, obtained as above described, were dialyzed for 12 days against running tap water, until the dialysate was practically free from chlorides. The light grayish precipitate of globulin was collected on a filter and washed with distilled water. It was redissolved in 10 per cent sodium chloride solution and was again precipitated by dialysis. After drying with alcohol and ether in the usual manner, the globulin weighed 1.66 gm. (*Globulin Preparation 22-A*). Carbon dioxide was passed into the original dialysate which contained the albumin, in order to precipitate any globulin that might have escaped separation by dialysis. After filtering off an insignificant quantity of precipitate, the

filtrate was heated in a boiling water bath until there was no further coagulation of protein. The very bulky coagulum was thoroughly washed with boiling water and dried with alcohol and ether (*Albumin Preparation 23-A*). The nearly pure-white albumin thus obtained weighed 7.83 gm.

*Albumin Preparation 25-B*, similarly prepared, was obtained in a yield of 7.53 gm.

(2). The salt extract of 500 gm. of bark was made 0.5 saturated with ammonium sulfate by addition of the solid salt, and the solution allowed to stand at room temperature for 24 hours. The precipitated protein was collected on a folded filter and redissolved by the addition of water, there being sufficient salts present in the moist precipitate to effect solution of the protein after dilution. After filtering off a small quantity of insoluble substance the clear filtrate was dialyzed until nearly free from sulfates and chlorides (9 days). The washed and dried globulin weighed 3.12 gm. (*Globulin Preparation 26-C*). The dialysate yielded *Albumin Preparation 27-C* (yield 8.27 gm.). *Albumin Preparation 29-D* and *Globulin Preparation 28-D* were similarly obtained in yields of 8.20 and 5.3 gm. respectively.

(3). Sufficient normal sodium hydroxide solution (15 cc.) to give a nearly neutral extract of 500 gm. of bark was added to 3 liters of 10 per cent sodium chloride solution. After the extraction the solution was made 0.5 saturated with ammonium sulfate. The precipitated protein was completely redissolved by addition of water, and the filtered solution dialyzed. Yields of 6.38 gm. of *Globulin Preparation 30-E* and 10.85 gm. of *Albumin Preparation 31-E* were obtained.

(4). *Globulin Preparation 32-F* and *Albumin Preparation 33-F* were obtained from a salt extract of 1 kilo of the bark. No sodium hydroxide had been added to the solvent. The proteins were precipitated from the salt extract by addition of ammonium sulfate to 0.5 of saturation. The precipitate was dissolved in water and the solution dialyzed. The globulin which separated was redissolved in 10 per cent sodium chloride solution and again precipitated by dialysis. After drying in the usual way it weighed 4.03 gm. The albumin obtained from the first dialysate amounted to 14.35 gm.

(5). *Globulin Preparation 34-G* and *Albumin Preparation 35-G* were obtained by extracting 1 kilo of bark with 6 liters of sodium chloride solution containing 30 cc. of normal sodium hydroxide. The extract was made 0.5 saturated with ammonium sulfate, the precipitated proteins were redissolved, and the salts removed by dialysis. The separated globulin weighed 9.20 gm., and 23.10 gm. of albumin were obtained from the dialysate.

(6). *Albumin Preparations 37-H* (10.15 gm.) and *39-J* (23.25 gm.), and *Globulin Preparations 36-H* (2.86 gm.) and *38-J* (9.83 gm.) were prepared from 1 kilo portions of bark as described in (5). The bark used for Preparations 36-H and 37-H was obtained from a tree, the vitality of which had been considerably impaired by age or disease, as indicated by the fact that some of its branches were dead. The relatively smaller yields obtained from this lot of bark may be ascribed to its inferior quality.

In these preparations the highest yield obtained of the albumin corresponded to 2.52 per cent of the moisture-free bark, and that of the globulin corresponded to 1.38 per cent.

By concentrating to a small volume the clear filtrates from the coagulated albumin and adding 2 volumes of 95 per cent alcohol, an appreciable quantity of a material was precipitated which had the properties of a proteose.

Data regarding the properties and composition of the globulin and of the proteose fraction will be presented in a subsequent communication.

The elementary composition of the several albumin preparations is given in Table II.

*Properties of the Albumin.*

The albumin, prepared as described, was obtained in a form which could be readily reduced to fine, light powder and was nearly pure white. Slight stirring or grinding caused it to become highly electrified. It can be precipitated from its solutions, either in water or in 10 per cent sodium chloride, by making the solution 0.44 saturated with ammonium sulfate. After having been thus precipitated, the moist albumin is still completely soluble in water or dilute salt solution. It coagulated when its aqueous or salt solutions were heated to 62–63°C. Preparations of the albumin were obtained having an ash content as low as 0.17 per cent, and which were free from phosphorus. When tested with Molisch's reagent, the albumin showed the presence of a small quantity of carbohydrates.

*Distribution of Nitrogen in the Albumin as Determined by the Van Slyke Method.*

The sample of albumin used for this analysis contained 0.17 per cent of ash and 15.02 per cent of nitrogen.

The analyses were made in duplicate in the usual way by boiling 3 gm. samples of the albumin for 30 hours with 20 per cent hydrochloric acid. The phosphotungstates of the bases were decomposed by means of a mixture of ether and amyl alcohol. The results of these analyses are given in Table III, IV, and V.

Table V shows the distribution of nitrogen in four groups, as

calculated from the results obtained by the Van Slyke method. The results are expressed in terms of percentages of the protein.

TABLE IV.

*Distribution of Nitrogen in the Locust Bark Albumin as Determined by the Van Slyke Method.**

Samples I and II, ash- and moisture-free, 2.7252 gm.; each containing 0.4093 gm. nitrogen.†

| Nitrogen. | I | II | I | II | Average. |
|---|---|---|---|---|---|
| | *gm.* | *gm.* | *per cent* | *per cent* | *per cent* |
| Amide N | 0.0383 | 0.0385 | 9.36 | 9.41 | 9.39 |
| Humin N adsorbed by lime | 0.0136 | 0.0138 | 3.32 | 3.37 | 3.34 |
| " " in ether-amyl alcohol extract | 0.0008 | 0.0005 | 0.20 | 0.12 | 0.16 |
| Cystine N | 0.0044 | 0.0043 | 1.08 | 1.05 | 1.07 |
| Arginine N | 0.0383 | 0.0387 | 9.36 | 9.46 | 9.41 |
| Histidine N | 0.0130 | 0.0127 | 3.18 | 3.10 | 3.14 |
| Lysine N | 0.0281 | 0.0288 | 6.87 | 7.04 | 6.96 |
| Amino N of filtrate | 0.2503 | 0.2488 | 61.15 | 60.78 | 60.96 |
| Non-amino N of filtrate | 0.0211 | 0.0219 | 5.15 | 5.35 | 5.25 |
| Total N regained | 0.4079 | 0.4080 | 99.67 | 99.68 | 99.68 |

* The nitrogen figures are corrected for the solubilities of the phosphotungstates of the bases.

† The nitrogen content of the protein was 15.02 per cent.

TABLE V.

*Distribution of Nitrogen in the Locust Bark Albumin as Calculated from the Van Slyke Analysis in Terms of Percentages of the Protein.**

| Nitrogen. | |
|---|---|
| | *per cent* |
| Amide | 1.41 |
| Humin | 0.53 |
| Basic | 3.09 |
| Non-basic | 9.95 |
| Total | 14.98 |

* Nitrogen content 15.02 per cent.

*Cystine, Tyrosine, and Tryptophane.*

The percentages of cystine and tyrosine in the albumin were estimated colorimetrically by the method of Folin and Looney (17). The estimation of tryptophane was made according to the method

of May and Rose (18), with certain modifications which have been described in a recent publication from this laboratory (19). The results of these analyses are included in Table III.

*Aspartic and Glutamic Acids.*

A quantity of the albumin (50 gm.) equivalent to 43.53 gm. of the ash- and moisture-free protein was hydrolyzed by boiling for 36 hours with 200 cc. of hydrochloric acid (specific gravity 1.1). The diamino acids were removed from the hydrolysate in the usual way by means of phosphotungstic acid, and the solution of monoamino acids concentrated to a small volume and saturated with hydrochloric acid gas. All of the concentrations in these determinations were made under reduced pressure and at a temperature not exceeding 40°C. in order to avoid the formation of pyrrolidone carboxylic acids. The crude glutamic acid hydrochloride was filtered off and washed with absolute alcohol which had been saturated with dry hydrochloric acid gas. The filtrate and washings were concentrated to a thick sirup. This was dissolved in about 400 cc. of water and treated with an excess of barium hydroxide. The barium salts of the dibasic amino acids were precipitated from the filtered solution by adding 4 volumes of 95 per cent alcohol. The precipitate was then dissolved in water and the barium removed with a slight excess of sulfuric acid. The chlorine was removed from the solution with silver sulfate. The silver was then removed with hydrogen sulfide and the sulfuric acid quantitatively removed with barium hydroxide. The solution containing the dibasic amino acids was concentrated to a small volume, and finally placed in a vacuum desiccator. The dry residue, after having first removed a small quantity of a sirupy material by triturating with glacial acetic acid, was dissolved in water, and the solution boiled with an excess of copper carbonate and filtered while hot. On cooling, the copper aspartate crystallized in the form of the characteristic microscopic sheaves.

After washing with cold water and drying in the air at room temperature, the copper aspartate weighed 6.77 gm., and contained 26.84 per cent of water of crystallization. This represents more nearly 4 molecules of water than the customary 4.5. After

heating for 2 hours at 130°C., the anhydrous product gave the following results on analysis.

0.1892 gm. substance: 0.0769 gm. CuO.
0.0868 " " required 4.5 cc. of 0.1 N acid.
$C_4H_5O_4NCu$. Calculated. Cu 32.7, N 7.19.
Found. " 32.5, " 7.28.

The above yield of crystalline copper aspartate corresponds to 3.36 gm. of aspartic acid, or 7.72 per cent of the albumin.

Copper was removed from the filtrate of the copper aspartate with hydrogen sulfide, and to the solution was added the crude glutamic acid hydrochloride which had been previously removed as already described. The total pure glutamic acid hydrochloride finally isolated in the usual way weighed 2.43 gm., equivalent to 1.95 gm. of the free acid, or 4.48 per cent of the albumin. The hydrochloride decomposed with effervescence at 198–199° and contained 7.49 per cent of nitrogen (theoretical 7.65 per cent).

## SUMMARY.

There have been isolated from the air-dried, inner bark of the common locust tree, *Robinia pseudacacia*, 2.52 per cent of albumin and 1.38 per cent of globulin (calculated on the basis of moisture-free bark). A significant quantity of a substance having the properties of a proteose was also obtained. Successive exhaustive extractions with distilled water, 10 per cent sodium chloride solution, 70 per cent alcohol, and 0.5 per cent sodium hydroxide removed from the coarsely ground bark 66.06 per cent of the total nitrogen, equivalent as protein (N × 6.25) to 11.56 per cent of the bark. The small quantities of nitrogen extracted by alcohol and alkali were non-protein in character, showing the absence in the bark of proteins of the prolamin or glutelin type.

By making a salt extract of the bark 0.44 saturated by addition of ammonium sulfate, practically all of the protein was precipitated. The mixed proteins thus obtained had enzymic properties, capable of decomposing urea and amygdalin. Working with the isolated proteins, the enzymes were found to be associated with the globulin and not with the albumin or proteose.

The albumin, either in aqueous or salt solutions, coagulated at 62–63°C. and was found to have the following percentage composi-

tion: C 54.52, H 6.83, N 14.77, and S 0.80. Analysis by the Van Slyke method showed it to have the following percentages of basic amino acids: arginine 4.39, histidine 1.74, lysine 5.45, and cystine 1.37. Colorimetric estimations of cystine, tyrosine, and tryptophane gave 1.03, 6.27, and 4.18 per cent respectively. There was obtained by actual isolation 7.72 per cent of aspartic acid and 4.48 per cent of glutamic acid.

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# PROTEINS OF THE COTTONSEED.*

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Ritthausen (1), in 1881, published a note on the separation of a protein by dialyzing the sodium chloride extract of the cottonseed. The protein thus prepared separated in the form of microscopic spheroids.

Later Osborne and Voorhees (2) published the results of a more thorough investigation of the proteins of the cottonseed. They obtained several preparations of a globulin, which, on elementary analysis, gave such closely agreeing results that they concluded that only one globulin is present in this seed. They also found that dilute potassium hydroxide solution removed considerable nitrogen from the cottonseed which had been previously extracted with sodium chloride solution to remove the globulin. No preparation of the alkali-soluble protein was obtained. A small quantity of a proteose was also isolated.

The globulin fraction of the cottonseed was later hydrolyzed and its amino acid content determined by Abderhalden and Rostoski (3).

Others (4, 5, 6) have analyzed, by the Van Slyke method, the mixture of total proteins extractable from cottonseed by various solvents, for the purpose of ascertaining the nutritive value of the cottonseed proteins as a whole.

In view of the fact that most other seeds examined contain several proteins, it was considered of interest to study the cottonseed to ascertain primarily whether or not proteins other than one globulin and a proteose could be isolated.

* A preliminary report of this paper was presented at the meeting of the Society for Experimental Biology and Medicine held in New York, January, 1925.



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*Preparation of the Material.*

The cottonseed[1] used in this investigation was grown in North Carolina, and was of the "Lone Star" variety. After hulling and delinting the seed, most of the oil was expressed by means of an Anderson expeller. Care was taken during the expression to avoid a temperature above 40–45°C. The finely ground press-cake was then extracted by percolation with benzene until the extract had only a pale yellow color. In this way most of the resinous and coloring matter, as well as fatty material, was removed. For this purpose benzene is far superior to ether. The percolation with benzene was followed by extraction with anhydrous ether. 100 gm. of the original press-cake yielded 73.9 gm. of air-dried extracted meal.[2] The air-dried material was powdered and sifted through a 40 mesh sieve. Analysis of the pale yellow material thus obtained gave the following results.

| | *per cent* |
|---|---|
| Moisture | 8.58 |
| Phosphorus (moisture-free basis) | 3.70 |
| Ash (moisture-free basis) | 8.24 |
| Nitrogen (ash- and moisture-free basis) | 10.37 |

*Extraction of the Meal.*

A series of preliminary experiments with sodium chloride solution was conducted to determine the optimum concentration for extracting the proteins from the meal. In these experiments a quantity of the air-dried extracted cottonseed meal containing 901 mg. of nitrogen was allowed to stand with 200 cc. of the solvent for 24 hours at room temperature with frequent shaking. The mixture was filtered and the nitrogen in the filtrate determined by the Kjeldahl-Gunning method. As shown in Table I, a 10 per cent solution of sodium chloride was found to be the concentration which extracted the most nitrogen.

Although a 10 per cent solution of sodium chloride proved to be the best solvent, a 20 per cent solution facilitated the separation of the extract from the residual meal. In the exhaustive extraction experiments, therefore, 20 gm. of the meal and 190 cc. of a 20 per cent sodium chloride solution were used for the first extraction. Extractions of the residue were then

[1] We are indebted to Mr. O. F. Cook, of the Bureau of Plant Industry, for the supply of this seed.

[2] This cottonseed product is referred to throughout this paper as *meal*, although the term does not conform to the official definition of cottonseed meal as adopted by the Association of Feed Control Officials of the United States.

continued with 10 per cent sodium chloride solution until the extracts were practically free from nitrogen. The extraction was hastened by shaking, and the extract was separated from the residue by centrifugation. The extract was brought each time to 200 cc., and the nitrogen content was determined in a filtered aliquot.

Digestion of the residue remaining from the extractions with sodium chloride solution, with 70 per cent alcohol, removed but a small quantity of nitrogen, which was found to be of a non-protein character, showing the absence of protein of a prolamin type. Exhaustive extraction of the residue was then continued at room temperature with 0.5 per cent sodium hydroxide solution. The results of these extraction experiments are summarized in Table II.

TABLE I.

*Extractions with Various Concentrations of Sodium Chloride Solution.*

| Concentration. | Nitrogen extracted. | |
|---|---|---|
| *per cent* | *gm.* | *per cent of total N* |
| 5 | 0.758 | 84.1 |
| 10 | 0.762 | 84.6 |
| 15 | 0.746 | 82.8 |
| 20 | 0.734 | 81.5 |

TABLE II.

*Nitrogen Extracted from the Cottonseed Meal by Various Solvents.*

| Nitrogen. | Nitrogen extracted. |
|---|---|
| | *per cent of total N* |
| Salt-soluble protein N | 76.6 |
| Alkali-soluble " " | 8.2 |
| Salt- and alkali-extractable non-protein N | 10.1 |
| Residual N (by difference) | 5.1 |

The non-protein nitrogen extractable from the meal by sodium chloride and by sodium hydroxide solutions amounted to 10.1 per cent of the total nitrogen. Tungstic acid was used as a protein precipitant, as applied by Folin and Wu (7) in blood analysis.

The high percentage of nitrogen extracted by sodium chloride solution is probably due to the application of benzene in preparing the meal, which eliminated practically all of the fatty, resinous, and coloring matter.

The salt extract became opalescent on heating to 52°C., and at 59° a flocculent precipitate separated. Further heating of the clear filtrate produced a coagulation at 69° and another at 83°C.

Osborne and Voorhees observed a somewhat similar behavior of the salt extract, but they did not investigate these coagula. The formation of a coagulum at a low temperature might lead one to suspect the presence of an albumin. The absence of an albumin in the cottonseed, however, has been definitely shown by Osborne and Voorhees (2), since they were unable to separate an albumin from the dialysate. We also were unable to find an albumin in the dialysate.

Two preparations having an unusual composition were obtained from the cottonseed meal. Although they separated from the saline extract of the meal at different temperatures, both preparations gave such closely agreeing results on analysis as to indicate

TABLE III.

*Composition of the High Ash-Yielding Fractions.*

| Determination. | Preparation I. | Preparation II. |
|---|---|---|
| | *per cent* | *per cent* |
| Moisture | 22.31 | 21.72 |
| Nitrogen (moisture- and ash-free basis) | 5.39 | 7.12 |
| Ash (moisture-free basis) | 68.20 | 67.52 |

Analysis of the ash:

| | *per cent* |
|---|---|
| $P_2O_5$ | 57.29 |
| CaO | 9.71 |
| MgO | 16.62 |
| $Na_2O$ | 13.90 |

that they were homogeneous and similar in composition. This substance yielded about 68 per cent of ash, which contained phosphorus equivalent to 57 per cent, calculated as $P_2O_5$. It also contained calcium, magnesium, and sodium. The nitrogen content of this material was between 5 and 7 per cent. The two fractions were obtained as follows: A clear, filtered sodium chloride extract of the meal was heated in a bath for a half hour at 62°C. A white coagulum resembling albumin separated. This was filtered off, washed with hot water, and dried (Preparation I).

On heating the filtrate for 1 hour at 85°C., a second precipitate separated, similar in appearance and properties to the first coagulum. This was filtered off and dried (Preparation II).

The manner in which these fractions separated as flocculent

coagula on heating at the stated temperatures would ordinarily have indicated an albumin and a globulin. The composition of these two fractions is given in Table III.

The alkali-soluble protein (glutelin) isolated represented a rather small fraction, amounting to only 0.73 per cent of the air-dried meal. It was obtained from the cottonseed residue, which had been exhaustively extracted with sodium chloride solution, by treatment with aqueous 0.2 per cent sodium hydroxide solution. The protein was precipitated from its filtered alkaline solution by bringing the latter to pH 4.4 with hydrochloric acid. It was thrice

TABLE IV.

*Protein Fractions Isolated per 100 Gm. of Cottonseed Meal.**

| Fractions isolated | Quantity isolated. | Nitrogen in the preparation. | |
|---|---|---|---|
| | *gm.* | *gm.* | *per cent of total N* |
| High ash-yielding fraction I | 0.83 | 0.01 | 0.1 |
| " " " II | 2.54 | 0.05 | 0.5 |
| α-Globulin | 2.59 | 0.43 | 4.9 |
| β-Globulin | 16.00 | 2.61 | 30.0 |
| Pentose protein | 2.08 | 0.22 | 2.6 |
| Glutelin | 0.73 | 0.09 | 1.0 |
| Total | 24.77 | 3.41 | 39.1 |

* All the figures are calculated on air-dried materials.

redissolved in sodium hydroxide and reprecipitated as before. This fraction had the following composition.

| | *per cent* |
|---|---|
| Carbon | 52.40 |
| Hydrogen | 6.27 |
| Nitrogen | 15.28 |
| Ash | 7.35 |
| Phosphorus | 0.35 |

Although dissolved and reprecipitated three times, the material still had a light brownish color and a high ash content.

The yields of the different purified proteins isolated are given in Table IV. Although the purified globulins isolated represented 34.9 per cent of the total nitrogen in the meal, it was found by direct dialysis of the salt extract that the total crude globulins which separated contained 59.9 per cent of the total nitrogen.

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The difference between the yields of the crude and purified globulins can be accounted for by losses incident to purification, such as denaturation, and incomplete precipitations on account of solubility. There is involved, however, another loss of a different nature. After removing from the sodium chloride extract of the cottonseed meal all the material coagulable by heat, there still remained in the filtrate an amount of nitrogen equal to 42.8 per cent of the total nitrogen present in the original extract, which leaves 57.2 per cent representing the nitrogen present in the coagulable fraction. This agrees fairly closely with the 59.9 per cent which separated by dialysis. The dialysate contained only 10.7 per cent of the total nitrogen, which corresponds to that of the pentose-protein fraction. This fraction is also included in the heat-uncoagulable fraction (42.8 per cent). As is shown in Table II, 10.1 per cent represents the non-protein nitrogen. Therefore, about 20 per cent of the total nitrogen, which is uncoagulable by heat, and which is lost during the dialysis, is unaccounted for. This fraction, however, is precipitable by tungstic acid. The isolation and identification of this material are under way.

### *The α- and β-Globulins.*[3]

By means of fractional precipitation with ammonium sulfate we have been able to separate two globulins from the cottonseed (8). One of these (α-globulin) was precipitated directly from a 10 per cent sodium chloride extract of the meal at 0.4 to 0.5 of saturation with ammonium sulfate. The other (β-globulin) flocculated at a saturation of 0.7 to 0.8, but only when the sodium chloride extract had been previously diluted with water, so that the ratio of the final volume to the weight of the original cottonseed meal extracted was 50:1.

The following procedure was used in the preparation of these globulins. 4 liters of a 20 per cent sodium chloride solution were added to 500 gm. of the meal and the mixture was stirred for 3 hours at room temperature.

[3] By α-globulin is meant a globulin precipitable by ammonium sulfate at a relatively low concentration as the first of two principal globulin fractions of clearly different chemical identity. The second globulin is, of course, designated the β-globulin. This terminology has been used in previous publications from this laboratory to designate globulins similarly obtained from various seeds.

After centrifugating and decanting the nearly clear supernatant solution, two subsequent extractions were made on the residue, using 3 and 4 liters of 10 per cent sodium chloride solution. The combined salt extracts contained 29.8 gm. of nitrogen, representing 68 per cent of the total nitrogen present in the meal. In order to eliminate all of the salt-soluble proteins from the residue before making the extractions with alkali to remove the glutelin, the residue from the salt extractions was again extracted four times with 3 liters of 10 per cent salt solution. These extracts were discarded so as to keep the volume within a workable limit.

The combined sodium chloride extracts (about 11 liters) were heated for 1 hour at 85°C. The precipitate, which represented the phospho compound, which has been already described, was removed, and the solution was made 0.23 saturated by the addition of ammonium sulfate. A flocculent precipitate, which quickly settled, separated. Most of the supernatant liquid was siphoned off, and the residue was centrifugated for a more thorough separation. The precipitate was dissolved in 0.1 saturated ammonium sulfate solution, filtered through paper pulp, and reprecipitated with ammonium sulfate at 0.33 saturation. The precipitate settled quickly and became brown on standing. For further purification it was again dissolved and precipitated with ammonium sulfate, and finally dissolved in 5 per cent sodium chloride solution, after which it was dialyzed for 10 days. The globulin separated in the form of brownish lumps. A small test sample readily dissolved in 10 per cent sodium chloride solution and coagulated at 95–97°C. The main portion, dehydrated in the usual way with alcohol and ether, formed a fine, light, tan-colored powder, and weighed 12.9 gm.

The supernatant liquid remaining from the first ammonium sulfate precipitation and which was 0.23 saturated with ammonium sulfate was diluted with distilled water to a volume of 25 liters, and made 0.74 saturated with ammonium sulfate. The $\beta$-globulin separated in large flocks, which soon settled. The clear supernatant liquid was siphoned off, and the precipitate was dissolved in 5 liters of 0.1 saturated ammonium sulfate solution. After having been filtered through paper pulp, the $\beta$-globulin was again precipitated by the addition of ammonium sulfate until the solution was 0.8 saturated. The purification process was repeated once more, after which the precipitate was dissolved in 2 liters of 5 per cent sodium chloride solution and dialyzed for 10 days. The slightly colored, grayish sediment dissolved readily in 10 per cent sodium chloride solution and coagulated at 92–93°C. The air-dried preparation weighed 75.97 gm. A sample of the dialysate yielded no precipitate, either when boiled or when saturated with carbon dioxide gas, showing that the globulins were completely removed.

Both globulin preparations were tested for pentose with Bial's reagent, but gave negative results.

The dialysate was concentrated under reduced pressure at a low temperature to a volume of 320 cc. and boiled for 15 minutes. The small amount of precipitate which separated was removed by filtration through paper pulp. 95 per cent alcohol was added to the clear filtrate until an

alcohol concentration of 70 per cent was reached. The material thus precipitated was washed with 95 per cent alcohol, redissolved in boiling water, filtered, and again precipitated from the slightly colored solution by the addition of 95 per cent alcohol as before.

TABLE V.

*Elementary Composition of the Isolated Cottonseed Proteins.**

| | α-Globulin. | β-Globulin. | Pentose protein. |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| C | 52.70 | 50.33 | 49.38 |
| H | 7.58 | 6.65 | 6.27 |
| N | 18.22 | 17.77 | 12.64 |
| S | 0.93 | 0.78 | |
| P | | | 0.194 |
| Pentose | None. | None. | 16.57 |
| Ash | 0.373 | 0.248 | 4.60 |

* Figures representing the elementary composition of the proteins were calculated on the basis of the ash- and moisture-free material, with the exception of those of phosphorus and ash, which were calculated on the moisture-free basis.

TABLE VI.

*Distribution of Nitrogen as Determined by the Van Slyke Method.**

| Nitrogen. | α-Globulin. | β-Globulin. | Pentose protein. |
|---|---|---|---|
| | *per cent* | *per cent* | *per cent* |
| Amide N | 11.40 | 11.70 | 12.99 |
| Humin N | 1.66 | 1.87 | 4.62 |
| Cystine N | 0.54 | 0.51 | 1.43 |
| Arginine N | 22.90 | 23.94 | 23.02 |
| Histidine N | 5.27 | 6.15 | 3.09 |
| Lysine N | 4.07 | 4.36 | 8.54 |
| Amino N of filtrate | 51.53 | 50.11 | 43.93 |
| Non-amino N of filtrate | 2.58 | 1.90 | 1.03 |

* Average results of duplicate analyses.

After drying with alcohol and ether, the material weighed 10.4 gm. This preparation gave a very strong pentose test with Bial's reagent. A quantitative determination of pentose by Tollen's phloroglucin method, using 0.5624 gm. of the air-dried material, gave 0.087 gm. of phloroglucid, which, calculated as pentose, represents 16.57 per cent of the ash- and moisture-free material. That the pentose is combined with the protein and not present as

an impurity is clear both from the method of preparation used and also from the large amount of pentose present. The elementary composition[4] of the proteins which have been isolated is given in Table V.

The distribution of nitrogen, as determined by the Van Slyke method (Table VI), does not disclose any noticeable difference between the $\alpha$-globulin and the $\beta$-globulin. The pentose protein (proteose fraction), on the other hand, was found to contain twice as much lysine as either of the globulins.

*Does the Cottonseed Contain Nucleic Acid?*

Osborne and Campbell (9) isolated from wheat embryo a nucleic acid, and later Levene and La Forge (10) confirmed their findings. It is but natural to assume that the embryos of other seeds also contain nucleic acid. The hulled cottonseed consists chiefly of embryonic tissue. The endosperm forms such a thin layer that it is distinguishable only under magnification. This, together with the high phosphorus content of the seed, suggested the presence of nucleic acid in the meal. The fact that we isolated two fractions from the meal, one yielding about 68 per cent of ash, which contained an amount of phosphorus equivalent to 57 per cent of $P_2O_5$, and the other (the pentose protein) containing 16.57 per cent of pentose, further justified an examination of the meal for nucleic acid, although on hydrolysis of nucleoproteins the pentose is usually found in the nucleic acid fraction, and not in the protein. In the attempt to isolate nucleic acid we followed in general the method used by Levene and La Forge for the preparation of triticonucleic acid. We examined not only the water extract of the meal but also the sodium chloride and the alkali extracts.

Although unable to isolate a nucleic acid, we obtained, from a 0.2 per cent potassium hydroxide extract of 5 kilos of meal, 0.78 gm. of a phosphorus-containing compound, which did not precipitate on adding picric acid to its solution slightly acidified with acetic acid, but did separate on the addition of hydrochloric acid to pH 2.0. It contained 2.3 per cent of organic phosphorus. Another phospho compound, which showed a constant phosphorus

[4] The carbon and hydrogen determinations were made by Mr. C. E. F. Gersdorff of this laboratory.

content of 1.55 per cent, was obtained, without applying peptic digestion, by precipitating the proteins with picric acid and then acidifying the clear filtrate with hydrochloric acid. The low phosphorus content of the small quantity isolated and the positive biuret test given by both of these preparations made it seem improbable that we were dealing with nucleic acid.


## SUMMARY.

By extracting finely ground cottonseed kernels (hull-free) with benzene, nearly all of the fatty and resinous substances and much of the coloring material are eliminated, thus facilitating the subsequent extraction of the proteins by different solvents. For this purpose benzene is far superior to ether.

Two globulins, designated as $\alpha$- and $\beta$-globulins, have been separated from a sodium chloride extract of cottonseed in yields of 2.59 and 16.00 per cent respectively. The $\alpha$-globulin was precipitated by the addition of ammonium sulfate to 0.4 to 0.5 saturation. It coagulated at 95–97°C. The $\beta$-globulin separated at a saturation of 0.7 to 0.8, but only when the extract had been previously diluted with water. It coagulated at 92–93°C.

Two preparations having similar properties and composition were separated from the sodium chloride extract by heating to 62° and 85°C. These fractions yielded 68.2 and 67.52 per cent of ash, which had the following percentage composition: $P_2O_5$, 57.29; CaO, 9.71; MgO, 16.62; and $Na_2O$, 13.90.

A pentose protein was also isolated (2.08 per cent), which contained 16.57 per cent of pentose, 0.194 per cent of phosphorus, and 12.64 per cent of nitrogen.

The distribution of nitrogen in the proteins isolated was determined according to the Van Slyke method.

A small quantity of a substance having the properties of a glutelin was also isolated.

An attempt to isolate a nucleic acid gave negative results.


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# A BIOCHEMICAL STUDY OF BONE GROWTH.

## II. CHANGES IN THE CALCIUM, MAGNESIUM, AND PHOSPHORUS OF BONE DURING GROWTH.

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The data given in this paper were obtained from the same bones on which the report of the age changes in ash, organic matter, and water was made (1). They constitute a second phase in the study of the chemistry of bone growth, in that they extend the picture to include the differential development of bone with respect to calcium, magnesium, and phosphorus incorporation. The source and distribution of the material and the methods of analysis used have been given in earlier papers (1–4).

Table I gives the means and the probable errors of the absolute amounts of the three elements as found in the humerus and femur of the male and female rats at the ages of 23, 30, 50, 65, 75, 100, and 150 days.

Since these elements are characteristic representatives of the ash of the bones, it is only to be expected that their general systemic and sex relations are similar to those exhibited by the ash and are similarly interpretable. A repetition thereof is accordingly unnecessary. It might be pointed out, however, that the greatest percentage increment during the growth period from 23 to 150 days of age is exhibited by calcium; phosphorus comes next; and magnesium last. This order is the order of decreasing atomic weight and may or may not mean anything.

Turning now from the absolute values which are chiefly useful as "standard" figures and as a basis for analytical computations, to the percentage values shown in Table II, one or two significant relations crop out.

The percentage of calcium, magnesium, and phosphorus of the femur is less than that of the humerus in both sexes at all ages.



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TABLE I.

*Observed Mean Calcium, Magnesium, and Phosphorus in the Humerus and Femur of Male and Female Albino Rats 23, 30, 50, 65, 75, 100, and 150 Days of Age.*

| Age. | Humerus. | | | Femur. | | |
|---|---|---|---|---|---|---|
| | Calcium. | Magnesium. | Phosphorus. | Calcium. | Magnesium. | Phosphorus. |
| | | | Male. | | | |
| *days* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| 23 | 3.41±0.13 | 0.091±0.002 | 1.80±0.07 | 3.99±0.17 | 0.108±0.004 | 2.14±0.09 |
| 30 | 5.11±0.21 | 0.144±0.005 | 2.66±0.11 | 7.22±0.36 | 0.213±0.010 | 3.85±0.17 |
| 50 | 8.75±0.27 | 0.237±0.006 | 4.45±0.13 | 15.45±0.53 | 0.433±0.010 | 7.89±0.26 |
| 65 | 17.63±0.71 | 0.415±0.011 | 8.89±0.34 | 34.51±1.59 | 0.810±0.033 | 17.69±0.76 |
| 75 | 19.94±0.41 | 0.474±0.008 | 9.93±0.21 | 39.55±0.81 | 0.933±0.015 | 19.92±0.36 |
| 100 | 26.30±1.06 | 0.600±0.027 | 12.91±0.54 | 54.45±2.77 | 1.258±0.077 | 27.03±1.36 |
| 150 | 41.75±1.59 | 0.946±0.040 | 20.64±0.81 | 90.69±4.24 | 2.028±0.093 | 45.26±2.00 |
| | | | Female. | | | |
| 23 | 3.67±0.14 | 0.095±0.004 | 1.90±0.07 | 4.60±0.21 | 0.123±0.006 | 2.42±0.11 |
| 30 | 4.77±0.16 | 0.133±0.004 | 2.49±0.09 | 6.87±0.30 | 0.186±0.009 | 3.62±0.15 |
| 50 | 9.81±0.20 | 0.249±0.007 | 4.94±0.13 | 17.60±0.54 | 0.451±0.006 | 8.76±0.23 |
| 65 | 15.88±0.64 | 0.410±0.010 | 7.82±0.30 | 31.04±1.36 | 0.764±0.024 | 15.53±0.61 |
| 75 | 18.97±0.48 | 0.441±0.007 | 9.43±0.28 | 38.36±1.09 | 0.869±0.025 | 19.33±0.55 |
| 100 | 27.57±0.90 | 0.605±0.011 | 13.47±0.34 | 57.99±2.00 | 1.291±0.038 | 28.56±0.95 |
| 150 | 34.64±1.15 | 0.778±0.035 | 16.89±0.59 | 73.25±2.73 | 1.634±0.070 | 36.12±1.39 |

TABLE II.

*Mean Percentage of Calcium, Magnesium, and Phosphorus in the Fresh Bones on Age.*

| Age. | Humerus. | | | Femur. | | |
|---|---|---|---|---|---|---|
| | Calcium. | Magnesium. | Phosphorus. | Calcium. | Magnesium. | Phosphorus. |
| | | | Male. | | | |
| *days* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 23 | 6.14±0.14 | 0.166±0.005 | 3.24±0.07 | 4.76±0.10 | 0.129±0.003 | 2.55±0.05 |
| 30 | 6.47±0.16 | 0.183±0.003 | 3.37±0.08 | 5.21±0.14 | 0.154±0.003 | 2.78±0.06 |
| 50 | 8.59±0.21 | 0.233±0.005 | 4.37±0.10 | 7.46±0.22 | 0.209±0.004 | 3.81±0.11 |
| 65 | 11.76±0.25 | 0.279±0.005 | 5.94±0.11 | 10.51±0.29 | 0.248±0.006 | 5.39±0.13 |
| 75 | 12.54±0.14 | 0.298±0.003 | 6.24±0.07 | 11.62±0.14 | 0.275±0.004 | 5.85±0.07 |
| 100 | 14.14±0.15 | 0.322±0.005 | 6.94±0.08 | 13.28±0.23 | 0.306±0.008 | 6.60±0.11 |
| 150 | 16.17±0.19 | 0.371±0.005 | 7.97±0.09 | 15.38±0.21 | 0.345±0.005 | 7.67±0.09 |
| | | | Female. | | | |
| 23 | 6.39±0.09 | 0.165±0.002 | 3.30±0.05 | 5.17±0.07 | 0.138±0.002 | 2.72±0.03 |
| 30 | 6.55±0.12 | 0.183±0.004 | 3.42±0.06 | 5.46±0.11 | 0.147±0.005 | 2.86±0.06 |
| 50 | 9.73±0.22 | 0.247±0.006 | 4.90±0.08 | 8.78±0.22 | 0.226±0.005 | 4.37±0.09 |
| 65 | 11.82±0.31 | 0.307±0.005 | 5.83±0.14 | 10.76±0.36 | 0.266±0.006 | 5.39±0.16 |
| 75 | 13.42±0.16 | 0.314±0.006 | 6.67±0.07 | 12.45±0.12 | 0.283±0.003 | 6.27±0.06 |
| 100 | 15.88±0.21 | 0.350±0.004 | 7.76±0.09 | 15.12±0.25 | 0.337±0.005 | 7.45±0.11 |
| 150 | 17.05±0.14 | 0.380±0.007 | 8.29±0.07 | 16.23±0.14 | 0.361±0.006 | 8.01±0.09 |

These relations parallel in direction those for the ash and are similarly interpretable.

The percentage of calcium in the bones of the female is at all ages greater than that of the male. This is not true of the per-

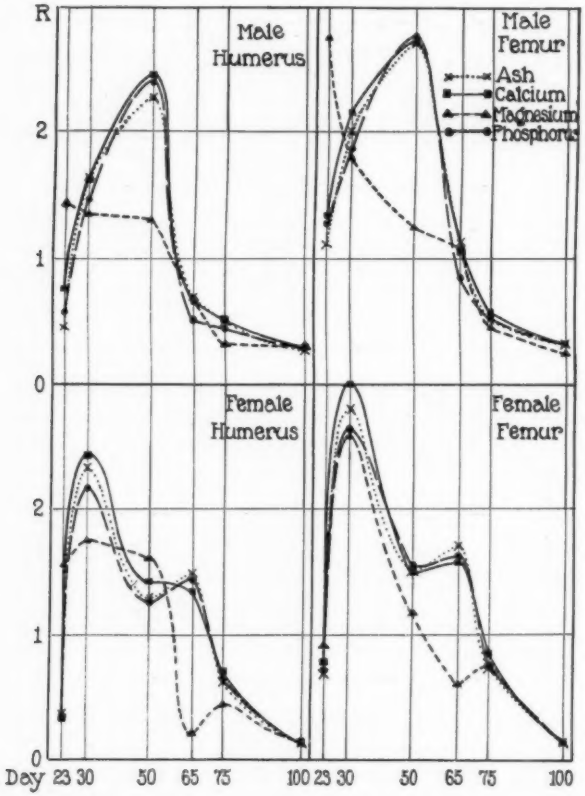


CHART 1. The rate of increment in percentage of calcium, magnesium, and phosphorus.

centage of magnesium or phosphorus. The mode of occurrence of these latter discrepancies indicates their reality. Their significance is obscure, but is not due to technical faults. It is evident

that the consistently greater percentage of ash in the bones of the female is primarily attributable to its calcium content.

A plotting of the rate of increment, on unit basis, of the percentage of calcium, magnesium, and phosphorus on age, as in

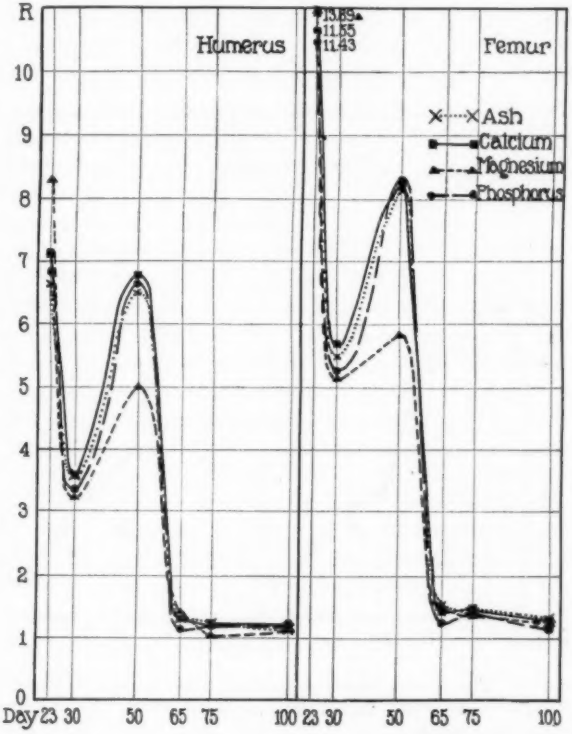


CHART 2. The growth capacity of the humerus and femur of the male rats on age for calcium, magnesium, and phosphorus together with that for ash for comparison.

Chart 1, brings out interesting relations. Similarly derived curves for the ash percentage are added for comparison.

In general the systemic and sex differences for calcium and phosphorus are like those exhibited by the ash percentage and

hence need no discussion. Magnesium, on the other hand, during the period of differentiation (from 23 to 75 days of age), deviates markedly from the course pursued by the ash and its chief constituents, calcium and phosphorus. This deviation is systemically uniform and sex-divergent in type. It is uncorrelatable with the course of the change in rate of increment of ash, organic matter, and water percentage. A detailed description or an interpretation of the divergencies in terms of the other factors

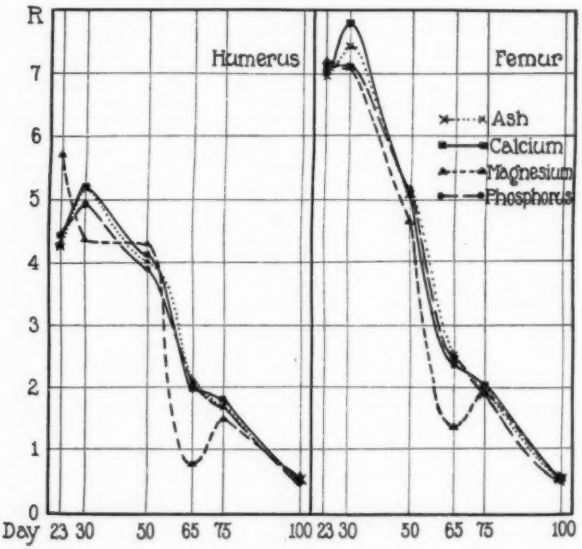


CHART 3. The growth capacity of the humerus and femur of the female rats on age for calcium, magnesium, and phosphorus together with that for ash for comparison.

is, therefore, unwarranted. All that can be said is that the processes concerned in the incorporation of magnesium into bone substance differ from those of the other constituents studied in their type relations to the general differential development of bone, and that magnesium as an ash component does not fit in with calcium and phosphorus with respect to their participation in ossification during the period of compositional differentiation.

A comparison of the age changes in rate of incorporation into bone substance of calcium, magnesium, and phosphorus, together with the relations of these to the rate of ash deposition brought to the unit basis of gm. per 100 gm. per day, serves to bring out the growth capacity relations of the bones for these constituents. The curves in Charts 2 and 3 give the data for the male and female groups respectively.

From these curves it is evident that in both bones of both sexes the growth capacity for calcium is essentially the same as that for phosphorus and ash at all ages. Therefore the interpretations of the age changes of ash, and the systemic and sex differences given in the preceding paper (1) apply to those for calcium and phosphorus and need no repetition.

The general conformity in degree of growth capacity for phosphorus with that for calcium indicates that the process of calcification is largely a matter of the formation of a calcium-phosphorus compound or compounds. The general conformity in degree of the growth capacity for calcium and phosphorus with that for ash indicates that the process of ossification is largely a matter of the deposition of this calcium-phosphorus compound.

It might be concluded from this that ossification and calcification are synonymous. Pragmatically the two terms are interchangeable; but from the point of view of the chemical differentiation of bone during growth this is not admissible, because of the fact that the growth capacity for magnesium is not the same as that for ash; because of the fact that the percentage of ash composition shifts with age as will be shown in a succeeding paper; and because of considerations already discussed in another connection (4).

From Chart 2 it is seen that the growth capacity for magnesium in the bones of the male is retarded as compared with that for calcium, phosphorus, and ash at 50 days of age. Chart 3 shows that the same occurs in the bones of the female at 65 days. These disturbances are attributable to puberal influences. They indicate that the course of magnesium incorporation into bone as an ash constituent differs from that of the ash as a whole and its chief components, calcium and phosphorus. Divergencies in the bones of the female at 23 and 30 days support this belief. The significance of this deviation is not evident. That the magnesium of

bone is different in its fundamental relation to bone ash composition from that of calcium is shown by these data and further from the fact that its percentage is increased in conditions of parathyroid deficiency while that of calcium tends to be decreased (5).

## SUMMARY AND CONCLUSION.

An analysis has been made of the chemical differentiation of the humerus and femur of male and female albino rats during the growth period from 23 to 150 days of age with respect to calcium, magnesium, and phosphorus.

Three significant relations have been brought out. The first is that the calcium percentage of the bones of the female albino rat is at all the observed ages greater than that of the male. The second is that in general the relations exhibited by calcium and phosphorus are the same as those which obtain for the ash and are similarly interpretable. The third is that the participation of magnesium, as an ash constituent, in the development of bone is different from that of calcium and phosphorus.

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# A BIOCHEMICAL STUDY OF BONE GROWTH.

## III. CHANGES IN THE COMPOSITION OF THE ASH DURING GROWTH.

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(Received for publication, May 7, 1925.)

Earlier reports, dealing with another line of investigation, have indicated that the percentage composition of bone ash shifts with age with respect to calcium, magnesium, and phosphorus (1, 2). The figures given in this paper establish the shift as a fact and permit an evaluation of its direction and significance.

Table I gives the means, and their probable errors, of the calcium, magnesium, and phosphorus of the ash of the bones at the stated ages. The values were derived from the data which served as the basis of the first two papers of this series (3, 4). They thus constitute a third phase in the study of the chemistry of bone growth, in that they extend the picture to include the differentiation which takes place in the ash of the bones during growth.

Before going on to the discussion of the changes incident to growth, the trends of systemic and sex differences may be noted.

There are no systemic or sex differences in the magnesium percentage of the ash.

There is a general tendency for the calcium percentage of the ash of the femur to be slightly less than that of the humerus. There is also a general tendency for the calcium percentage of the ash of the bones of the male to be less than that of the female. The relation is reversed at 30 and 75 days of age. This reversal coincides with periods of disturbance in differential development of bone, as already discussed in detail (3). It indicates that these disturbances are reflected in ash composition in as far as calcium is concerned.

The phosphorus percentage of the ash tends to be slightly greater in the femur than in the humerus, and in the bones of the male than

in those of the female. It is noteworthy that, contrary to *a priori* expectation, the direction of systemic and sex differences in phosphorus percentage is the opposite of that of calcium. This indicates that the phase of metabolism in which calcium is utilized in bone growth, is not completely representative of the phosphorus metabolism.

The tendency towards the presence of a higher percentage of

TABLE I.

*Mean Percentage of Calcium, Magnesium, and Phosphorus in the Humerus and Femur of Male and Female Albino Rats on Age.*

| Age. | Humerus. | | | Femur. | | |
|---|---|---|---|---|---|---|
| | Calcium. | Magnesium. | Phosphorus. | Calcium. | Magnesium. | Phosphorus. |
| | | | Male. | | | |
| *days* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* | *per cent* |
| 23 | 35.93±0.09 | 0.97±0.02 | 18.98±0.03 | 35.74±0.09 | 0.97±0.01 | 19.18±0.04 |
| 30 | 36.75±0.05 | 1.04±0.01 | 19.16±0.07 | 36.22±0.11 | 1.07±0.01 | 19.40±0.09 |
| 50 | 36.57±0.10 | 0.99±0.01 | 18.62±0.05 | 37.05±0.09 | 1.05±0.02 | 18.94±0.06 |
| 65 | 37.30±0.08 | 0.89±0.02 | 18.86±0.05 | 37.01±0.15 | 0.88±0.01 | 19.04±0.04 |
| 75 | 37.24±0.09 | 0.89±0.01 | 18.53±0.04 | 36.87±0.07 | 0.87±0.01 | 18.57±0.04 |
| 100 | 37.40±0.04 | 0.85±0.01 | 18.34±0.07 | 37.32±0.06 | 0.86±0.01 | 18.54±0.05 |
| 150 | 37.50±0.11 | 0.85±0.02 | 18.51±0.08 | 37.49±0.10 | 0.84±0.02 | 18.62±0.08 |
| | | | Female. | | | |
| 23 | 36.59±0.09 | 0.95±0.01 | 18.88±0.07 | 36.11±0.07 | 0.97±0.001 | 18.99±0.07 |
| 30 | 36.47±0.09 | 1.02±0.02 | 19.03±0.06 | 36.08±0.07 | 0.97±0.002 | 19.06±0.06 |
| 50 | 36.84±0.17 | 0.94±0.02 | 18.60±0.10 | 37.25±0.16 | 0.96±0.002 | 18.60±0.08 |
| 65 | 37.48±0.15 | 0.98±0.02 | 18.51±0.06 | 37.20±0.19 | 0.93±0.001 | 18.70±0.08 |
| 75 | 37.05±0.05 | 0.87±0.02 | 18.41±0.05 | 36.82±0.08 | 0.84±0.001 | 18.55±0.04 |
| 100 | 37.83±0.07 | 0.84±0.01 | 18.47±0.03 | 37.66±0.08 | 0.84±0.001 | 18.57±0.04 |
| 150 | 37.58±0.10 | 0.83±0.02 | 18.32±0.11 | 37.64±0.08 | 0.84±0.002 | 18.57±0.08 |

phosphorus in the bones of the male and in the femur is correlatable with the fact that the bones of the male contain more organic matter than do those of the female, and the fact that the femur has more organic matter than the humerus. It may be, therefore, that these systemic and sex differences in phosphorus percentage of the ash are to be attributed to the differences in organic matter.

Although the differences just noted are small in degree, the consistency of their occurrence makes them suggestive.

Turning now to the changes in ash composition with age, it is

seen from the table that the calcium percentage of both bones of both sexes increases with age to a statistically valid degree. The greater part of the increase in the bones of the male occurs during the growth period from 23 to 65 days of age. Thereafter but little change is seen, though a drift upwards is indicated. In the female, while the greater part of the increase also occurs during the period from 23 to 65 days, stabilization is not evident until 100 days of age. These sex differences are concordant with those exhibited by the ash as a whole, as has been discussed in an earlier paper (3). They are similarly interpretable. The significant point is that the differentiation of ash with respect to its calcium content is directed by the same sex differential factors of influence as regards time of action during growth as is the differentiation of the bone as a whole with respect to its growth by increments of ash.

The percentage of magnesium in the ash of both bones of both sexes decreases to a statistically valid degree during the growth period from 23 to 150 days of age. The greater part of the decrease in the bones of the male takes place between 50 and 65 days of age, and in the female between the ages of 65 and 75 days. Thereafter the changes are inconsiderable. This sex difference in time of decrease and stabilization is comparable with that which is exhibited by the water of the bones. For if the magnesium percentage values be expressed as a rate of change in percentage on unit basis, it is found that the time relations of the dip and recovery in the curve are quite the same as those for the water percentage calculated on the same basis (3). This being so, it is possible that there is an association of some sort between the changes in magnesium percentage of the ash and the water percentage of the bones, and the rôle of the sex-specific factors therein.

The percentage of phosphorus in the ash of both bones of both sexes decreases to a statistically valid degree during the growth period from 23 to 150 days of age. The greater part of the decrease is completed by the time the rats are 75 days of age. Thereafter the changes are inconsiderable.

Phosphorus and calcium are thus seen to change in opposite directions, contrary to *a priori* expectation. This may be interpreted as indicating a change in the type of Ca-P complex laid down with age, or an intramolecular change with age, or as an expression of a difference in type metabolism in which an associa-

tion of phosphorus with organic matter increments is present as distinct from the association of phosphorus with calcium in the process of calcification. The ultimate interpretation must rest on further data.

The significant point of these findings is that the greater part of the shift in ash composition during growth, with respect to calcium, magnesium, and phosphorus, takes place during the period when the chemical differentiation of the bone as a whole leading to the structure which is sex- and systemically characteristic of the adult from the chemical point of view, is taking place, and that the incidence of stabilization at the end of puberty is coterminous with that of the stabilization of chemical differentiation of the bone as a whole.

SUMMARY.

The percentage composition of the ash of the humerus and femur of male and female albino rats with respect to calcium, magnesium, and phosphorus changes during the growth period from 23 to 150 days of age.

The changes are practically complete at 75 days of age. That is to say by the end of the puberal period.

The changes consist of an increase in the calcium and a decrease in the magnesium and phosphorus percentage of the ash.

Acknowledgement should be made to Miss Elizabeth Stow for assistance in the computations on which these studies are based.

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# ANIMAL CALORIMETRY.

Twenty-Ninth Paper.

## ON FAT PRODUCTION IN A YOUNG HOG.

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(Received for publication, May 12, 1925.)

Work reported a year ago from this laboratory by Rapport, Weiss, and Csonka (1) described the production of fat from starch at a maximum rate of 82 gm. daily in a young hog weighing 28 kilos. Since the growth of this hog had been stunted by protein restriction and by the administration of large and often toxic doses of benzoate of soda, it was thought that a young, normally growing hog might show an even better fat-producing power. It was also intended to keep a strict record of the nitrogen balance in the growing animal and thereby attain a more complete picture of the factors underlying the growth process. Unfortunately, the animal frequently urinated while in the calorimeter, and when very large amounts of carbohydrate were administered it vomited in its cage, thereby contaminating the urine. However, the records obtained are of interest as showing very high respiratory quotients and a large production of fat from carbohydrate.

### *Methods.*

The *diet* of the hog during the experimental period consisted in the daily administration at 5 p.m. of 700 cc. of milk (3.85 gm. N), 4 gm. of bone ash, 4 gm. of the salt mixture of Mendel used by Karr (2), containing Ca, Mg, Fe, KI, and I, 1 gm. of Harris yeast vitamin powder, and commercial corn-starch between 300 and 700 gm. In the later experiments 100 gm. of dry milk powder (5.60 gm. N and 43.5 gm. lactose) were added to the diet.

* Fellow in Medicine of The Rockefeller Foundation.

On account of the tendency to restlessness in the young animal its body temperature could not be determined during the calorimeter periods and hence only the "heat eliminated" could be measured and compared with the heat by the indirect method of calorimetry. This is not as accurate as when the gain or loss of body heat may be computed from the changes of rectal temperature.

TABLE I.

*Basal Metabolism of Hog II.*

Surface = $9\sqrt[3]{\text{Weight}^2}$

| Experiment No. | Date. | Age. | Weight. | Surface. | Urine N per hr. | R.Q. | Heat production. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Per hr. | Per day. | Per sq. m. of surface. |
| | *1924* | *days* | *kg.* | *sq. m.* | *gm.* | | *cals.* | *cals.* | *cals.* |
| 1 | Dec. 17 | 70 | 8.5 | 0.375 | 0.044 | 0.93 | 16.8 | 403 | 1,076 |
| 2 | " 22 | 75 | 9.5 | 0.404 | 0.044 | 0.95 | 18.8 | 451 | 1,118 |
| 3 | " 24 | 77 | 10.0 | 0.418 | 0.044 | 0.90 | 19.0 | 456 | 1,092 |
| | *1925* | | | | | | | | |
| 7 | Jan. 12 | 96 | 12.3 | 0.480 | 0.037 | 0.87 | 21.1 | 506 | 1,056 |
| 20 | Feb. 2 | 117 | 18.1 | 0.620 | 0.048 | 0.79 | 27.9 | 670 | 1,080 |
| Average | | | | | | | | | 1,085 |
| Difference | | | | | | | | | 3 per cent |

The *basal metabolism* was determined following a day on which the only diet was a liter of whole milk given at 5 p.m.

The *calculations* for the production of fat from carbohydrate were made according to the method described in the paper by Rapport, Weiss, and Csonka. 1 liter of extra $CO_2$ elimination above the non-protein respiratory quotient of unity equals a deposition of 1.7 gm. of fat derivable from 4.6 gm.[1] of glucose or 4.1 gm.[1] of starch and is accompanied by the liberation of 0.8 calorie.

*Basal Metabolism.*

Table I presents the basal metabolism of Hog II from the 70th to the 117th day of its age. During this period the weight of the

[1] These figures were erroneously given in the former paper, but do not invalidate any calculations therein. For this method consult also Lusk (3).

hog more than doubled, but the basal heat production per square meter of surface remained the same, thereby affording additional proof of the truth of this fundamental biological principle.

Hog I (of Rapport, Weiss, and Csonka) showed a metabolism of 1,035 calories per square meter of surface when 78 days old and weighing 20 kilos, but this value fell gradually, probably on account of a lack of the protein element in the dietary.

*Protein Metabolism.*

The hog showed a great avidity for the milk protein given in his diet. When the hog was about 100 days old the nitrogen of the protein ingesta was increased from 3.85 gm. daily to 9.45 gm., with the result that the urine nitrogen increased only from 0.99 to 1.17 gm. However, when the nitrogen in the diet was increased to 15 gm. the urinary nitrogen rose to 4.32 gm., indicating that a surplus of protein was being provided at a time when approximately 10 gm. of nitrogen were being deposited as protein in the animal.

The diets contained:

| Composition of the diet. | Total N. |
|---|---|
| | gm. |
| Starch, 300 to 700 gm.; milk 700 cc. | 3.85 |
| " 300 " 700 " " 700 " milk powder, 100 gm. | 9.45 |
| " 300 " 700 " " 700 " " " 200 " | 15.05 |

Table II indicates the results.

It is evident that the hog had a pronounced capacity for converting milk protein into its own flesh. Kelly (4) has recently shown that a growing hog weighing 40 kilos may retain 11 to 12 gm. of nitrogen daily during a period of more than 6 weeks.

*Fat Production.*

The hog, which had received a diet containing all the starch it would take at 5 p.m. the evening before, was given 200 gm. of starch and 100 gm. of glucose at 10 a.m. and was at once placed in the calorimeter for observation. Only hours during which the animal was quietly resting could be used. A summary of the results is presented in Table III.

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TABLE II.

*Table of Food Nitrogen and Urine Nitrogen per Day.*

| Date. | Age. | N in diet. | Urine N. | | |
|---|---|---|---|---|---|
| | | | Average. | Maximum. | Minimum. |
| *1924–25* | *days* | *gm.* | | | |
| Dec. 17, Jan. 5, 11, 15 | 70–99 | 3.85 | 0.99 | 1.05 | 0.93 |
| Jan. 16 to 22 | 100–106 | 9.45 | | | |
| " 23 " 27 | 107–111 | 9.45 | 1.16 | 1.21 | 1.13 |
| " 30 | 114 | 9.45 | 1.17 | | |
| " 31 | 115 | 5.50* | 1.15 | | |
| Feb. 1 | 116 | 9.45 | 1.47 | | |
| " 2 | 117 | 5.50* | | | |
| " 3 | 118 | 15.05 | 1.88 | | |
| " 4 to 10 | 119–125 | 15.05 | | | |
| " 11 | 126 | 15.05 | 4.32 | | |
| " 12 | 127 | 20.70 | | | |
| " 13 | 128 | 9.45 | | | |
| " 14 | 129 | 9.45 | 5.71 | | |
| " 15 | 130 | 9.45 | 3.10 | | |
| " 16 | 131 | 9.45 | 3.02 | | |

* 1 liter of milk the only diet.

TABLE III.

*Effect on Hog II of Giving 200 Gm. of Starch and 100 Gm. of Glucose.*

(Assumed basal metabolism, 21.1 calories.)

| Experiment No. | Date. | Age. | Weight. | Time after food. | Urine N per hr. | R.Q. | Fat produced from carbohydrate per hr. | Heat production. | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Per hr. | Increase over basal. |
| | *1925* | *days* | *kg.* | *hrs.* | *gm.* | | *gm.* | *cals.* | *per cent* |
| 11 | Jan. 16 | 100 | 14.7 | 3, 4 | 0.039 | 1.52* | 6.9 | 41.9 | |
| 13 | " 20 | 104 | 15.1 | 2, 4, 5, 6 | 0.039 | 1.44 | 6.2 | 43.8 | |
| 16 | " 22 | 106 | 15.2 | 2 | 0.047 | 1.34 | 4.8 | 42.6 | |
| 17 | " 23 | 107 | 16.3 | 2, 3 | 0.047 | 1.37 | 5.2 | 42.4 | |
| Average | | | | | | | 5.8 (= 55.1 cals.) | 42.7 | 102 |

* R. Q.: 3rd hour, 1.58; 4th hour, 1.46.

The average results found in these four experiments show that the specific dynamic action of glucose caused a doubling of the heat production, the greatest ever observed under like conditions. The quantity of fat produced from carbohydrate averaged 5.8 gm. hourly and contained 55.1 calories. During these early hours after carbohydrate ingestion the hog therefore manifested the following relations:

| Energy factors during fat production. | Energy per hr. | In terms of total. | In terms of basal. |
|---|---|---|---|
| | *cals.* | *per cent* | *per cent* |
| Basal metabolism | 21.1 | 22 | |
| Specific dynamic action of carbohydrate | 21.6 | 22 | 102 |
| Fat produced from carbohydrate | 55.1 | 56 | 261 |
| Total energy liberated or transformed | 97.8 | 100 | |

TABLE IV.

*Metabolism of Hog II 20 Hours after the Ingestion of 700 Gm. of Corn-Starch.*

| Experiment No. | Date. | Age. | Weight. | Time after food. | Urine N per hr. | R.Q. | Fat produced from carbohydrate per hr. | Heat production. | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Per hr. | Increase over basal. |
| | *1925* | *days* | *kg.* | *hrs.* | *gm.* | | *gm.* | *cals.* | *per cent* |
| 9 | Jan. 14 | 98 | 13.2 | 20 | 0.046 | 1.29 | 2.9 | 30.4 | |
| 10 | " 15 | 99 | 13.6 | 19, 20 | 0.041 | 1.41 | 4.1 | 31.0 | |
| Average | | | | | | | 3.5 | 30.7 | 45 |

It is evident that a hog, when given carbohydrate in quantity, may not only double its own heat production, but also transform carbohydrate into fat at a rate which represents a caloric equivalent of two and a half times the basal heat requirement.

The respiratory quotients obtained during these periods are the highest ever recorded. The only existing experiments directly comparable with these were performed 6 years ago by F. G. Benedict. Here quotients of 1.48 were obtained from geese stuffed with grain according to the method by which *pâtés de foie gras* are produced in Strassbourg. These experiments, known to this laboratory since their accomplishment, are referred to with the friendly permission of Dr. Benedict.

TABLE V.

*Details of Some Experiments on Hog II.*

| Experiment No. | Date. | Time. | $CO_2$ | $O_2$ | R.Q. | $H_2O$ | Urine N. | Non-protein. $CO_2$ | Non-protein. $O_2$ | Non-protein. R.Q. | Calories. Protein. | Calories. CH to fat. | Calories. Non-protein. | Calories. Indirect. | Calories. Eliminated. | Fat from CH. | Weight of hog. | Diet. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *1925* | *p.m.* | *gm.* | *gm.* | | *gm.* | *gm.* | *gm.* | *gm.* | | | | | | | *gm.* | *kg.* | |
| 7 | Jan. 12 | 12–1 | 7.55 | 6.15 | 0.89 | 9.02 | 0.037 | 7.20 | 5.84 | 0.90 | 0.98 | | 20.13 | 21.11 | 22.88 | | 12.3 | Basal. |
| | | 1–2 | 7.22 | 6.22 | 0.84 | 7.80 | 0.037 | 6.89 | 5.91 | 0.85 | 0.98 | | 20.12 | 21.10 | 21.49 | | | |
| | | | | | | | | | | | | | | 42.21 | 44.37 | | | |
| 8 | " 13 | 12–1 | 20.11 | 9.60 | 1.52 | 16.91 | 0.046 | 19.68 | 9.22 | 1.55 | 1.21 | 2.85 | 32.57 | 36.63 | 39.54 | 6.1 | 13.0 | 400 gm. corn-starch + 60 gm. glucose at 10 a.m. |
| | | 1–2 | 18.78 | 9.19 | 1.49 | 14.30 | 0.046 | 18.35 | 8.81 | 1.52 | 1.21 | 2.54 | 31.13 | 34.88 | 36.08 | 5.4 | | |
| | | 2–3 | 18.57 | 9.76 | 1.38 | 14.69 | 0.046 | 18.14 | 9.38 | 1.41 | 1.21 | 2.14 | 33.14 | 36.49 | 36.41 | 4.5 | | |
| | | 3–4 | 16.05 | 8.01 | 1.46 | 13.12 | 0.046 | 15.62 | 7.63 | 1.49 | 1.21 | 2.09 | 26.96 | 30.26 | 34.47 | 4.4 | | |
| | | | | | | | | | | | | | | 138.26 | 146.50 | 20.4 | | |
| 10 | " 15 | 11–12 | 16.52 | 8.50 | 1.41 | 9.13 | 0.041 | 16.14 | 8.16 | 1.44 | 1.08 | 2.00 | 28.83 | 31.91 | 28.25 | 4.3 | 13.6 | 19 and 20 hrs. after corn-starch, 700 gm. |
| | | 12–1 | 16.42 | 8.00 | 1.40 | 8.84 | 0.041 | 15.04 | 7.66 | 1.43 | 1.08 | 1.84 | 27.06 | 29.98 | 28.30 | 3.9 | | |
| | | | | | | | | | | | | | | 61.89 | 56.55 | 8.2 | | |
| 13 | " 20 | 11.20–12.20 | 28.75 | 15.02 | 1.39 | 20.81 | 0.039 | 28.38 | 14.69 | 1.40 | 1.04 | 3.33 | 51.90 | 56.27 | 47.61 | 7.1 | 15.1 | 200 gm. corn-starch + 100 gm. glucose at 10 a.m. |
| | | 1–2 | 24.19 | 12.47 | 1.41 | 21.75 | 0.039 | 23.82 | 12.14 | 1.43 | 1.04 | 2.90 | 42.89 | 46.83 | 49.20 | 6.2 | | |
| | | 2–3 | 21.92 | 10.99 | 1.45 | 20.08 | 0.039 | 21.55 | 10.66 | 1.47 | 1.04 | 2.81 | 37.66 | 41.51 | 48.07 | 6.0 | | |
| | | 3–4 | 17.02 | 8.01 | 1.55 | 20.89 | 0.039 | 16.65 | 7.68 | 1.58 | 1.04 | 2.48 | 27.13 | 30.65 | 34.54 | 5.3 | | |
| | | | | | | | | | | | | | | 175.26 | 179.42 | 24.6 | | |

It was noted by Rapport, Weiss, and Csonka that, after giving starch at 5 p.m. to a hog, the respiratory quotients of the animal were always above unity on the following day and that the basal metabolism remained at a high level. Table IV presents similar results obtained upon Hog II.

It is evident that 20 hours after starch ingestion the heat production may be 45 per cent above the basal, and a respiratory quotient of 1.41 indicates the continuance of a high level of fat production, which in one instance reaches 4.1 gm. per hour.

*Additional Details.*

While the limitations as to the full publication of all the details of these experiments are compelling, still it seems necessary to present in full the results found in given types of the work, and to this end four experiments have been selected and are given in Table V.

During the twelve hourly periods here reported the heat calculated by the indirect method was 417.6 calories and the "heat eliminated" (body temperature changes disregarded) was 426.8 calories, a difference of only 2.2 per cent, which affords a good check as to the reliability of the calculated heat production.

It will be noted that in Experiment 7 protein metabolism yielded 4.6 per cent of the calories during a period when the basal metabolism was determined and this proportion was reduced to 2.1 per cent of the calories following a large ingestion of starch and glucose.

Attention is called to the striking increase in the $CO_2$ elimination following the ingestion of starch. Sometimes the respiration resembled dyspnea. In Experiments 8 and 13, 6 hours after the ingestion of starch with glucose, the metabolism reached the same level found in Experiment 10 made 20 hours after the ingestion of starch. The respiratory quotients remained quite uniformly high throughout these hours.

In Table VI an attempt has been made to estimate the metabolism of the hog per day, and this is represented graphically in Fig. 1.

Fig. 1 shows the total heat production of the animal in its relation to the transformation of starch into fat. The heat pro-

duction from protein was not far from 3 per cent of the total heat produced and is here neglected.

TABLE VI.

*Miscellaneous Computations.*

Hog II. Age, 100 days. Weight, 13.5 kilos.

| | Average per day. | Maximum per day. |
|---|---|---|
| Basal metabolism, *cals* | 506 | |
| First 6 hrs. after starch | 256 | |
| Next 18 hrs. after starch | 553 | |
| Estimated metabolism after starch | 809 | |
| Specific dynamic action of starch, *per cent* | 60 | |
| | Average. *gm.* | Maximum. *gm.* | | |
| Fat retention, first 6 hrs | 34.8 | 41.4 | | |
| " " next 18 hrs | 63.0 | 83.8 | | |
| Estimated fat production, *gm* | | | 98 | 125 |
| " " " per kg. body weight, *gm* | | | 7 | 9 |
| " calories retained as fat | | | 931 | 1,093 |
| Fat production in terms of basal metabolism, *per cent* | | | 184 | 216 |
| " " " " " hog's metabolism, *per cent* | | | 115 | 135 |
| (*a*) Starch required for production of 98 gm. fat, *gm* | | | 239 | |
| (*b*) " for heat requirement of hog, *gm* | | | 197 | |
| | | | 436 | |
| Deduct 5 per cent of (*a*) which appears in (*b*) | | | 12 | |
| Total starch requirement, *gm* | | | 424 | |

TABLE VII.

*Alcohol Checks.*

| Experiment No. | Date. | R.Q. | Calories per hr. | |
|---|---|---|---|---|
| | | | Indirect. | Direct. |
| | *1924* | | | |
| 193 | Dec. 15 | 0.671 | 31.18 | 29.75 |
| | *1925* | | | |
| 194 | Jan. 27 | 0.669 | 24.40 | 24.82 |
| 195 | " 30 | 0.664 | 23.03 | 24.42 |
| Average | | 0.668 | 26.20 | 26.33 |

It is evident that a well nourished animal partaking liberally of carbohydrate may construct fat from it to the extent of nearly 1 per cent of its own body weight daily.

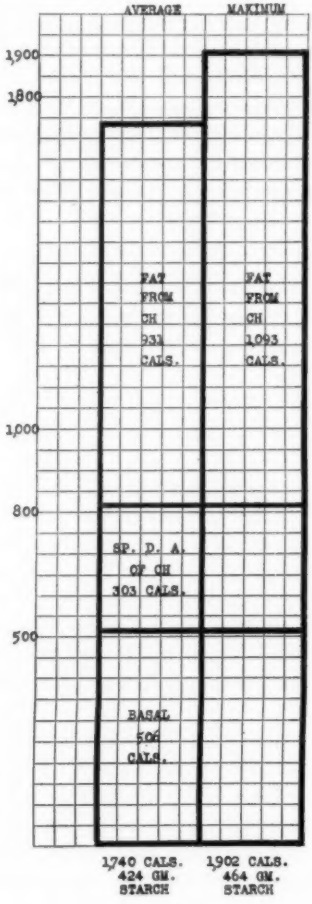


FIG. 1.

It has already been pointed out in work from this laboratory that the height of metabolism is not proportional to the height of

the respiratory quotient, and this point is again confirmed. The production of fat from starch takes place with a loss of only 5 per cent in the energy content of the starch. The secret of how, out of the turmoil of the intermediary metabolites, a hog may produce from starch 2 mg. of fat *per second*, is a question for the physiological chemist of today. The cumulative sum of such reactions leads to the production of 7.1 gm. of fat *per hour*, which was found to be the maximum in the series.

Table VII presenting tests of the respiration calorimeter, performed during the period during which these experiments were conducted, conveys a general idea as to the reliability of the apparatus.

## CONCLUSIONS.

1. The basal metabolism of a growing hog from the 70th to the 117th day of its life, during which time it increased in weight from 8.5 to 18.1 kilos, averaged 1,085 calories per day within a ± variation of 3 per cent.

2. The hog showed a continued capacity for retaining milk protein in large amounts when milk and starch formed the basis of the diet.

3. When starch and glucose were given together on the morning of a day following a large ingestion of starch, the metabolism rose 100 per cent above the basal and the production of fat from carbohydrate amounted to a storage of caloric energy in the form of fat equal to 260 per cent of the basal metabolism.

4. Estimated on a 24 hour basis, the hog has a capacity of using ingested carbohydrate so that more than half of the material is employed for fat construction and less than one-half for his quiet resting metabolism.

5. After giving starch to a hog the respiratory quotients were maintained at about 1.40 during at least 20 hours. The highest quotient obtained, which is the highest ever recorded, was 1.58.

6. During twelve hourly periods the heat calculated by the indirect method was 417.6 calories, and the "heat eliminated" by the hog (body temperature changes disregarded) was 426.8 calories, a difference of only 2.2 per cent, which affords a justification of the validity of the calculations.

7. Though the production of fat from carbohydrate did not

exceed 2 mg. *per second*, yet in 24 hours the rate of production reached at times 125 gm. *per day*, or 0.9 per cent of the hog's weight.

The authors wish to express their deep gratitude to Professor Graham Lusk for his help and guidance in accomplishing these experiments.

Acknowledgment is made to the Corn Products Refining Company for their kindness in supplying us with the pure crystalline glucose used in these experiments.

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# ON THE RELATION OF THYROID SECRETION TO SPECIFIC DYNAMIC ACTION.*

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As a result of researches of this laboratory on the regulation of metabolism and from a survey of the literature on specific dynamic action of foods, we had reason to believe that the thyroid gland was an *essential* factor in this phenomenon. We held this view in spite of the fact that the literature on the specific dynamic action of food in various thyroid diseases quite uniformly indicated that there was no change from the normal in myxedema in which there is an undoubted diminished activity of the thyroid gland or in Graves' syndrome, where, in certain stages of the disease, there is an increased function of the gland. Thus during the last decade six investigators from as many laboratories report no change from normal in Graves' disease, myxedema, or "thyrogenous obesity" (Du Bois (1), Aub and Means (2), Undeutsch (3), Rolly (4), Plaut (5), and Liebesny (6)). Weiss and Adler (7), however, in one case of Graves' disease find an increase in specific action but in a single case of myxedema they also find an increase. More to the point is an experiment by Lusk (8) in which he determined the specific dynamic action of meat in a phlorhizinized thyroidectomized dog, 19 days after thyroid removal and on the 12th day of diabetes. He found a large increase in heat production over the basal level.

Several of the above investigators have found a uniformly lowered specific dynamic action in patients suffering of pituitary disease (Rolly (4), Plaut (5), and Bernhardt (9)). When desiccated thyroid is fed to such patients an increased specific dynamic action is observed. Abelin (10, 11), in animal experiments, had also temporarily obtained increased specific dynamic action by feeding thyroid and several other drugs (phenylethylamine, adrenalin, etc.).

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* A preliminary report of some of these experiments was made before the Society for Experimental Biology and Medicine on May 24, 1924 (Baumann, E. J., and Hunt, L., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 447). In it it was erroneously stated that in three of these rabbits no thyroid tissue was found. More careful microscopic examination of the thyroid area has since revealed the presence of very small amounts of hyperplastic thyroid (0.1 and 1 mm. respectively) in two of them.



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If the thyroid were essential for specific dynamic action to manifest itself after food intake, *a completely thyroidectomized* animal should show no specific dynamic action. If the thyroid is not a factor then there should not be much difference in specific dynamic action before and after thyroidectomy.

*Methods.*

Experiments were planned in which this problem was studied in the way just indicated. We chose rabbits for the experimental animal because it is possible, though very difficult, completely to remove their thyroid glands without interfering with the inferior parathyroids. The greatest disadvantage of using rabbits for metabolism experiments in which a basal level of metabolism is at times desired, lies in the fact that it is difficult to empty completely their alimentary canals, even after a 4 day fast and yet have the animal in an otherwise physiological condition. However, we found that after a 36 to 48 hour fast, their respiratory quotients were almost always below 0.74 which we regarded as satisfactory for our purpose. From the standpoint of obtaining a basal metabolic rate, a cat or dog would be a far superior animal to work with, but only very rarely would one find a dog or cat with a sufficient number of parathyroid glands so favorably located that they could remain with their blood supply intact, while the thyroids were removed. And even in the few animals that might be found in which such operations could be successfully performed, it would be still more difficult to dissect the parathyroids without leaving a few thyroid cells attached. Realizing the extraordinary capacity of thyroid tissue for regeneration, it seemed that the chances of ever obtaining a complete uncomplicated thyroidectomy in the carnivores were small. Moreover, dogs almost always have accessory thyroid tissue in the thymus area and elsewhere, while this is much less common in rabbits.

The rabbits were fed on a diet of alfalfa (or clover) hay and oats. Carrots, lettuce, or other fresh vegetables were added three or more times a week. They were fasted 36 to 48 hours before metabolism studies were made. Bread was usually substituted for the hay ration on the day before the fast was to begin; it is probably absorbed more rapidly than hay. The specific dynamic

action experiments on the same animal were made at intervals of not less than 1 week so that recovery from the fast might be complete.

The metabolism was determined by the Haldane method (12) with the modified apparatus used in previous studies from this laboratory. The $CO_2$ output and $O_2$ intake were determined for a 2 hour period before, and for two 2 hour periods beginning $\frac{1}{2}$ hour after the administration of glucose.

Except in a few experiments the N excretion was not determined, and consequently not taken into account in calculations of the energy produced. This was simply calculated from the $CO_2$ excreted and the respiratory quotient. This mode of calculation introduces an error of about 1 per cent. Another source of error was disregarded in these experiments. No correction was made for gases arising from intestinal fermentation. This has been estimated to amount to from 2 to 8 cc. per kilo per hour in the rabbit (13) and would cause an error of less than 1 per cent at the most. As a result of a 2 day fast, the contents of the stomach, where most of the fermentation occurs, should be greatly reduced in amount and the fermentation occurring should be correspondingly lessened. It is estimated that the combined errors from these sources would be considerably less than 5 per cent; such errors could not mask our data to any serious extent in these experiments.

No mechanical record was kept of the movements of the animals. All of the rabbits were trained and had previously been used for other metabolism studies for at least 2 or 3 months and often for more than 6 months. Animals that cannot be trained are not used. Objective notes of the behavior of the animals during the experiments were kept and where the animals were active or irritable, experiments were discarded. This did not happen after thyroidectomy. The rabbits were placed in the metabolism chambers at least 15 minutes before the experimental period was begun.

*Presentation of Data.*

The specific dynamic action of 15 gm. of glucose was determined five times in four normal rabbits and eleven times in seven thyroidectomized rabbits at various intervals after thyroidectomy (12 to 86 days).

The specific dynamic action of 25 gm. of glucose was determined ten times in six normal rabbits and thirty-one times in

TABLE I.

*Specific Dynamic Action of 25 Gm. of Glucose in Normal Rabbits.*

| No. | Sex. | Weight. | $CO_2$ expired in 2 hrs. | $O_2$ consumed in 2 hrs. | R.Q. | Total calories in 2 hrs. | Total extra calories. |
|---|---|---|---|---|---|---|---|
| | | *gm.* | | | | | |
| 467 | M. | 2,515 | 2.90* | 2.86 | 0.74 | 9.43* | |
| | | | 4.08 | 3.91 | 0.76 | 12.99 | |
| | | | 3.90 | 3.14 | 0.90 | 10.86 | +4.99 |
| 467 | " | 2,376 | 2.98 | 2.99 | 0.72 | 9.80 | |
| | | | 3.57 | 3.02 | 0.86 | 10.30 | |
| | | | 4.02 | 3.53 | 0.83 | 11.93 | +2.63 |
| 472 | " | 2,388 | 3.11 | 3.14 | 0.72 | 10.01 | |
| | | | 4.19 | 3.77 | 0.81 | 12.86 | |
| | | | 4.20 | 3.48 | 0.88 | 11.91 | +4.75 |
| 472 | " | 2,507 | 2.92 | 2.94 | 0.72 | 9.71 | |
| | | | 4.02 | 3.75 | 0.78 | 12.53 | |
| | | | 4.15 | 3.24 | 0.93 | 11.27 | +4.38 |
| 474 | " | 2,548 | 2.97 | 2.92 | 0.74 | 9.66 | |
| | | | 4.22 | 3.52 | 0.87 | 12.07 | |
| | | | 4.47 | 3.51 | 0.93 | 12.14 | +4.89 |
| 474 | " | 2,615 | 2.89 | 2.95 | 0.71 | 9.72 | |
| | | | 4.26 | 3.72 | 0.83 | 12.64 | |
| | | | 4.45 | 3.72 | 0.87 | 12.73 | +5.93 |
| 477 | " | 2,746 | 3.54 | 3.66 | 0.70 | 12.06 | |
| | | | 4.89 | 5.10 | 0.70 | 17.38 | |
| | | | 4.68 | 4.18 | 0.81 | 14.16 | +7.42 |
| 477 | " | 2,746 | 3.56 | 3.66 | 0.71 | 11.97 | |
| | | | 4.84 | 4.54 | 0.78 | 15.09 | |
| | | | 5.22 | 4.01 | 0.95 | 13.94 | +5.09 |

* 2 hour fore period followed by two 2 hour experimental periods after feeding glucose.

six thyroidectomized animals from 8 to 411 days after thyroidectomy.

Five control experiments in which water at 38°C. was given instead of glucose were also made on one normal and four thyroidectomized animals. The metabolism during the 2 hour periods, ½ to 4½ hours after giving water, is often below the 2 hour control period. The maximum increase observed for any animal was 0.38 calorie.

In normal rabbits the specific dynamic action of 15 gm. of glucose was found to vary from 1.31 to 3.81 extra calories, the average of the five experiments in four rabbits being 2.23 calories. The specific dynamic action of 25 gm. of glucose varied from 2.63 to 7.42 extra calories over the control period. The average of the ten experiments on six different animals was 5.13 calories. Seven of the ten experiments gave values beween 4.38 and 5.93 extra calories. Some of these experiments including the two showing the greatest deviation from the average are given in Table I as examples.

Following thyroidectomy marked changes in the specific dynamic action of glucose were observed. In general there was a gradual decrease to a level far below the normal. In five of the ten thyroidectomized animals studied, 25 gm. of glucose showed no specific dynamic action after varying periods. In one of these (No. 388) careful macroscopic and microscopic examinations of the thyroid area showed no thyroid tissue. In three other animals (Nos. 398, 443, and 469) a small thyroid area was found in each case, while in the remaining rabbit (No. 468) considerable thyroid tissue was found. In this case thyroid exhaustion was promoted by a pregnancy as well as by the low iodine content of the ration.[1] As an example of this group, the experiments on Rabbit 443 are presented in Table II. No thyroid tissue was noted on gross dissection of the thyroid area. Microscopic examination of the larynx, however, revealed a tiny thyroid fragment measuring about 0.1 mm. in diameter. In each of the five rabbits whose specific dynamic action *decreased*

[1] We have frequently noted spontaneous goiters of various degrees, usually mild, in the rabbits bred in our laboratory even though we are located on the sea coast. The spontaneous goiters are most marked in pregnant animals. This is no doubt due to the low iodine content of the main items of the dietary—alfalfa hay and oats—and to the rigorous exclusion of any form of volatile iodine from the animal quarters.

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but was not *entirely obliterated* regenerated hyperplastic thyroid tissue was found on gross examinations and confirmed histologically.[2]

Besides showing that the extent of specific dynamic action is,

TABLE II.

*Showing the Disappearance of Specific Dynamic Action of Glucose Following Thyroidectomy.*

Rabbit 443 ♀.

| Glucose given. | After thyroidectomy. | Weight. | $CO_2$ expired in 2 hrs. | $O_2$ consumed in 2 hrs. | R.Q. | Total calories in 2 hrs. | Total extra calories. |
|---|---|---|---|---|---|---|---|
| *gm.* | *days* | *gm.* | | | | | |
| 15 | 0 | 2,480 | 3.83* | 3.73 | 0.75 | 12.32* | |
| | | | 4.02 | 3.48 | 0.84 | 11.81 | |
| | | | 4.38 | 4.37 | 0.73 | 14.40 | +2.08 |
| 14 | 12 | 2,644 | 3.63 | 3.54 | 0.75 | 11.68 | |
| | | | 4.15 | 3.45 | 0.87 | 11.87 | |
| | | | 4.85 | 3.87 | 0.91 | 13.39 | +1.90 |
| 25 | 0 | 2,532 | 3.50 | 3.46 | 0.74 | 11.38 | |
| | | | 4.92 | 4.12 | 0.87 | 14.07 | |
| | | | 5.17 | Lost. | 0.89 | 14.53 | +5.84 |
| 25 | 19 | 2,541 | 3.14 | 2.90 | 0.79 | 9.69 | |
| | | | 4.18 | 3.40 | 0.89 | 11.75 | |
| | | | 4.75 | 3.61 | 0.96 | 12.39 | +4.76 |
| 25 | 76 | 2,816 | 4.15 | 3.93 | 0.77 | 13.07 | |
| | | | 4.75 | 3.75 | 0.92 | 13.01 | |
| | | | 5.07 | 3.93 | 0.94 | 13.65 | +0.58 |

* 2 hour fore period followed by two 2 hour experimental periods after feeding glucose.

Adult rabbit. Removed right and left thyroid lobes Oct. 31, 1923. Killed Feb. 18, 1924. 24 days after last specific dynamic action experiment. Autopsy. Death due to extensive pneumonia involving both lungs. No thyroid tissue found on gross examination of larynx and thymus area. Microscopic examination of larynx showed minute thyroid fragment, 0.1 mm. in diameter.

[2] In the strain of rabbits bred at Montefiore Hospital, the thyroid gland is closely adherent to the thyroid cartilage and for complete removal, one must dissect very closely. Even if this be successfully accomplished, if a few thyroid cells are loosened, they will often transplant anywhere in the wound with the utmost ease.

within certain limits, dependent on the amount of thyroid tissue present, they are excellent control animals for those in which no specific action occurred. The amount of thyroid tissue necessary for some specific dynamic action is very small, certainly not more than 10 per cent of the amount ordinarily present and probably less. The outstanding point of these experiments, then, is, that without thyroid tissue the administration of glucose does not cause any specific dynamic action. In the presence of a small amount of thyroid tissue—less than 10 per cent of that normally present—one will observe for a long time a little specific dynamic action after feeding glucose though much less than is found in normal animals.

In the completely thyroidectomized animal (No. 388), it took 65 days before the specific dynamic action of glucose was completely abolished, while in Rabbits 443, 398, 469, and 468, in which some thyroid fragments were found, 75, 110, 257, and 281 days respectively elapsed before this happened (see Tables II, III, and IV).[3] From these facts we conclude that it takes about 65 days for the thyroid secretion in the tissues to become exhausted. This is in accord with what is known about the action of thyroid substance. For example, Boothby and Sandiford (14) from a study of the rate at which the metabolism of a thyroidectomized person decreased after administration of thyroxin have calculated that the life of thyroxin in the body may be about 70 days.

We regarded this fact of such importance that we attempted to verify it in two other ways and these efforts were fairly successful. A rabbit (No. 398) whose specific dynamic action had been completely abolished (in fact following the feeding of glucose there was a lower heat production than in the control period) was fed 0.1 gm. of an active preparation of desiccated thyroid the 2nd, 3rd, and 5th days before determining the specific dynamic action of 25 gm. of glucose. The specific dynamic action was then found to be about 1 extra calorie. The extra heat produced was probably greater than this for in the four previous experiments made on this animal over a period of 10 weeks, there were

[3] In these four animals the length of time required for the disappearance of specific dynamic action was roughly proportional to the amount of thyroid tissue found at autopsy.

TABLE III.

*Showing Disappearance of Specific Dynamic Action of Glucose Following Thyroidectomy and the Influence of Thyroid Feeding.*

Rabbit 398♂.

| Glucose given. | After thyroidectomy. | Weight. | $CO_2$ expired in 2 hrs. | $O_2$ consumed in 2 hrs. | R.Q. | Total calories in 2 hrs. | Total extra calories. | Remarks. |
|---|---|---|---|---|---|---|---|---|
| *gm.* | *days* | *gm.* | | | | | | |
| 15 | 0 | 2,542 | 3.19* | 3.29 | 0.71 | 10.87* | | |
| | | | 4.28 | 4.01 | 0.78 | 13.34 | | |
| | | | 4.10 | 3.17 | 0.94 | 11.04 | +2.64 | |
| 15 | 12 | 2,753 | 2.88 | 2.92 | 0.72 | 9.58 | | |
| | | | 3.20 | 3.24 | 0.72 | 10.64 | | |
| | | | 3.30 | 2.94 | 0.82 | 9.89 | +1.37 | |
| 25 | 19 | 2,876 | 2.70 | 2.62 | 0.75 | 8.69 | | Urinated. Began experiment over again. |
| | | | 4.61 | 3.50 | 0.96 | 12.22 | | |
| | | | 5.13 | 3.83 | 0.97 | 13.49 | +8.33 | |
| 25 | 77 | 3,200 | 2.97 | 2.96 | 0.73 | 9.77 | | |
| | | | 3.98 | 3.16 | 0.92 | 10.90 | | |
| | | | 4.43 | 3.62 | 0.89 | 12.45 | +3.81 | |
| 25 | 110 | 3,101 | 3.32 | 3.36 | 0.72 | 11.04 | | |
| | | | 3.44 | 2.94 | 0.85 | 10.02 | | |
| | | | 3.55 | 2.96 | 0.87 | 10.15 | −1.91 | |
| 25 | 117 | 3,085 | 3.18 | 3.17 | 0.73 | 10.46 | | |
| | | | 3.44 | 2.86 | 0.87 | 9.84 | | |
| | | | 3.55 | 2.72 | 0.95 | 9.48 | −1.60 | |
| 25 | 131 | 2,935 | 3.32 | 3.44 | 0.70 | 11.31 | | |
| | | | 3.37 | 3.27 | 0.75 | 10.84 | | |
| | | | 3.43 | 2.55 | 0.98 | 8.95 | −2.83 | |
| 25 | 215 | 2,337 | 3.75 | 3.92 | 0.70 | 12.78 | | Gave 0.1 gm. desiccated thyroid, 2nd, 3rd, and 5th days previous. |
| | | | 4.44 | 4.08 | 0.79 | 13.78 | | |
| | | | 4.43 | 3.59 | 0.90 | 12.34 | +1.00 | |
| 25 | 238 | 2,632 | 2.58 | 2.75 | 0.68 | 8.80 | | Calculated with R.Q. of 0.70. |
| | | | 2.88 | 2.54 | 0.82 | 8.63 | | |
| | | | 3.44 | 2.62 | 0.95 | 9.19 | +0.39 | |

TABLE III—*Concluded.*

| Glucose given. | After thyroidectomy. | Weight. | $CO_2$ expired in 2 hrs. | $O_2$ consumed in 2 hrs. | R.Q. | Total calories in 2 hrs. | Total extra calories. | Remarks. |
|---|---|---|---|---|---|---|---|---|
| *gm.* | *days* | *gm.* | | | | | | |
| 25 | 372 | 2,539 | 2.92 | 2.93 | 0.72 | 9.71 | | |
| | | | 3.05 | 3.02 | 0.73 | 10.03 | | |
| | | | 3.10 | 2.72 | 0.83 | 9.20 | +0.32 | |
| 25 | 411 | 2,490 | 2.71 | 2.51 | 0.79 | 8.36 | | Gave 25 mg. KI on the 9th and 11th days previous. |
| | | | 2.82 | 2.47 | 0.83 | 8.37 | | |
| | | | 3.09 | 2.51 | 0.90 | 8.61 | +0.26 | |

* 2 hour fore period followed by two 2 hour experimental periods after feeding glucose.

Adult male rabbit, Oct. 31, 1923, age 14 months. Removed right and left thyroid lobes. Beginning Oct. 15, 1924 a gradual loss of weight amounting to 700 gm. was noted which was arrested for a few months. Jan. 7, 1925 found in semicomatose condition. Killed (14 months after thyroidectomy). Autopsy at once. Weight 2,086 gm. One flattened thyroid mass, 2 mm. in diameter on right side which on histological examination proved to be very hyperplastic thyroid containing no colloid. Kidneys both alike, equal in size, enlarged, edematous, and surface pitted. Ureters enlarged and thickened. Bladder walls also thickened. Bladder contained a large egg-shaped calculus, weight 23 gm. Other organs negative. Coma probably due to uremia

produced about 2 calories *less* in the 4 hours after administration of either 50 cc. of 50 per cent glucose solution or 50 cc. of water at 38°C. than would have been expected if the basal rate obtained on the first 2 hours of the same day had been maintained in the 4 following hours, so that probably 3 extra calories were produced by the glucose in this experiment.

This animal was killed when found in a semicomatose condition some 6 months later. On autopsy it was found to have a very large calculus in the bladder which in turn had probably caused the degenerative changes that were found in the kidneys. It had a small hyperplastic thyroid fragment but this was apparently unable to supply sufficient thyroid secretion to cause any specific dynamic action and this supposition received ample confirmation in the results of the specific dynamic action experiment following thyroid feeding which we have just described. The pertinent experiments on this animal are presented in Table III.

The thyroid fragments found in the incompletely thyroidectomized rabbits were usually only a few mm. in size and always very hyperplastic. These regenerated bits when examined histologically contained practically no colloid material, the medium in which the active substance of the thyroid gland is stored. When an animal having an hyperplastic gland is fed with any form of iodine, Marine and Rogoff (15) have shown that the gland is involuted and colloid begins to appear in the alveoli in about 48 hours.

If an animal, whose specific dynamic action had decreased to less than half of the normal amount after incomplete thyroid removal, were fed some iodine and that iodine were utilized by the thyroid gland an increased thyroid secretion would be expected and the specific dynamic action should be increased if our hypothesis be correct. In one animal, Rabbit 468, in which the presence of thyroid tissue was suspected and in which on autopsy two fragments, $5 \times 3$ and $2 \times 2$ mm., respectively, were found, this was tried twice and each time an increased specific dynamic action of 25 gm. of glucose resulted temporarily.

The specific dynamic action of 25 gm. of glucose in this animal dropped to 1 calorie 40 days after the thyroid glands were removed. 75 and 82 days after thyroidectomy the extra heat produced in 4 hours after giving 25 gm. of glucose had increased and was each time found to be 2.5 calories. A week later a control experiment showed that no extra heat was produced when 50 cc. of water at 38°C. were given by stomach tube under the same conditions as those under which the other experiments were made. On the 2 days following the control experiment, 50 mg. of KI were given by mouth (78 mg. of iodine in all). The specific dynamic action of 25 gm. of glucose was determined again 35 days after the iodine feeding and it was found to have increased from 2.5 to 3.9 extra calories.

The rabbit was bred shortly after this experiment in connection with some other studies. On the 20th day of pregnancy (the period of gestation of the rabbit is 30 days) 25 gm. of glucose were found to cause an extra heat production of only 0.7 calorie—1.8 calories less than the specific dynamic action had been *before* the administration of KI.

The fact that thyroid hyperplasias may occur during preg-

nancy is well known. An increased demand for thyroid secretion during pregnancy is therefore indicated and we believe that the lowered specific dynamic action at this time undoubtedly resulted from thyroid exhaustion which occurs under these conditions. The animal became pregnant again during the next 6 months after which the specific dynamic action of 25 gm. of glucose was only 0.6 calorie. Some time after this experiment (on December 4 and 6), the rabbit was given 25 mg. of KI on each day by mouth (a total of 39 mg. of iodine). On January 12, the specific dynamic action of 25 gm. of glucose was 3.6 calories, an increase of 3 calories over the previous result. These experiments are given in Table IV.

It is possible that the increase in specific dynamic action caused by the administration of 50 mg. of KI in one case and 100 mg. in the other may have been due to the iodine acting through some other path than the thyroid gland, but we think such a possibility extremely unlikely. The first time KI was given, the specific dynamic action remained increased more than 30 days after, and this is in perfect agreement with what is known concerning the action of thyroid secretion. Thyroid secretion must behave like a catalyst and be capable of taking part in chemical reactions of the body over and over again in order for so small an amount of material to remain active as long as we know it does. On the other hand, iodides, unless taken up by the thyroid, are promptly excreted. They can be detected in the urine in less than 15 minutes after taking some by mouth and practically all is excreted in 24 hours. It is improbable that 38 mg. of iodine would act 30 days after feeding through any channel other than the thyroid. So these observations on Rabbit 468 are regarded as furnishing strong support to our thesis; *viz.*, thyroid secretion is necessary for specific dynamic action.

Two other animals (Nos. 398 and 469) (see Table III) that were given 50 mg. of KI after the specific dynamic action of 25 gm. of glucose had dropped to practically nothing showed no increase in specific dynamic action. At autopsy a small amount of thyroid tissue was found and on microscopic examination no colloid was visible. It is possible that thyroid tissue that has been functioning at its greatest capacity, as these fragments must have been, for so long a time (about

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TABLE IV.

*Showing Decrease of Specific Dynamic Action of Glucose Following Partial Thyroidectomy and the Influence of Feeding KI.*

Rabbit 468 ♀.

| Glucose given. | After thyroidectomy. | Weight. | $CO_2$ expired in 2 hrs. | $O_2$ consumed in 2 hrs. | R.Q. | Total calories in 2 hrs. | Total extra calories. | |
|---|---|---|---|---|---|---|---|---|
| *gm.* | *days* | *gm.* | | | | | | |
| 25 | 26 | 2,834 | 2.86* | 2.89 | 0.72 | 9.51* | | |
| | | | 3.93 | 3.19 | 0.90 | 10.95 | | |
| | | | 3.88 | 2.98 | 0.95 | 10.36 | +2.29 | |
| 25 | 40 | 2,804 | 3.16 | 3.16 | 0.73 | 10.39 | | |
| | | | 3.69 | 3.40 | 0.79 | 11.39 | | |
| | | | 3.84 | 3.01 | 0.93 | 10.43 | +1.04 | |
| 25 | 75 | 2,911 | 3.02 | 2.95 | 0.74 | 9.82 | | |
| | | | 3.79 | 3.21 | 0.86 | 10.94 | | |
| | | | 4.18 | 3.25 | 0.94 | 11.25 | +2.55 | |
| 25 | 82 | 2,950 | 3.17 | 3.04 | 0.76 | 10.04 | | |
| | | | 3.90 | 3.39 | 0.84 | 11.46 | | |
| | | | 4.04 | 3.23 | 0.91 | 11.15 | +2.53 | |
| 0 | 89 | 2,897 | 3.18 | 3.05 | 0.77 | 10.12 | | 50 cc. $H_2O$ at 38°C. by sound. |
| | | | 3.04 | 3.30 | 0.67 | 10.36 | | |
| | | | 3.14 | 2.92 | 0.78 | 9.79 | +0.24 | |
| 25 | 124 | 2,880 | 3.35 | 3.19 | 0.76 | 10.66 | | Fed 50 mg. KI 34th and 35th days previous. |
| | | | 3.84 | 3.74 | 0.75 | 12.35 | | |
| | | | 4.26 | 3.82 | 0.81 | 12.89 | +3.92 | |
| 25 | 145 | 3,020 | 3.48 | 3.57 | 0.71 | 11.70 | | Pregnant 20th day of gestation. |
| | | | 3.84 | 3.74 | 0.75 | 12.35 | | |
| | | | 3.57 | 3.20 | 0.81 | 10.80 | +0.65 | |
| 25 | 281 | 2,832 | 3.11 | 3.28 | 0.69 | 10.60 | | Calculated with R.Q. of 0.70. |
| | | | 3.50 | 3.23 | 0.79 | 10.81 | | |
| | | | 3.76 | 3.22 | 0.85 | 10.95 | +0.56 | |
| 25 | 357 | 2,663 | 3.40 | 3.44 | 0.72 | 11.31 | | Fed 25 mg. KI 37th and 38th days previous. |
| | | | 4.25 | 3.82 | 0.81 | 12.86 | | |
| | | | 4.72 | 3.92 | 0.88 | 13.38 | +3.62 | |

* 2 hour fore period followed by two 2 hour experimental periods after feeding glucose.

Jan. 30, 1924. Removed right and left thyroid lobes. Rabbit 8 months old. Killed 10 weeks after last specific dynamic action experiment. In good condition, weight 2,913 gm. Approximately 21 months old. Autopsy findings negative except for a large active regenerated thyroid fragment on left side of trachea, 5 × 3 mm., and a smaller thyroid fragment on right side of trachea.

9 and 13 months in these animals) could have become exhausted and was no longer capable of secreting its hormone even when finally supplied with the "bausteine" it requires. Something of this nature occurs in long standing cases of myxedema in man. Medication with iodine will not increase the metabolism of such patients nor relieve their symptoms, but thyroid gland will, just as feeding of thyroid to Rabbit 398 was effective in restoring the specific dynamic action of glucose though KI failed. Too little is yet known about absolute exhaustion and atrophy of thyroid tissue to pass on this point. The possibility that thyroid secretion is but one of the necessary links in the highly complicated chain of events that lead to specific dynamic action and that thyroidectomy may cause changes in other organs essential for this phenomenon must not be overlooked. We do not believe these negative experiments detract from the positive ones that were described first nor that they supply any evidence against our hypothesis.

In several of the experiments, a low respiratory quotient was obtained in the first period after glucose feeding and in four instances this was associated with a considerable increase in heat production; *e.g.*, Experiment 1, Table I; Experiment 2, Table III; Experiment 6, Table IV. We think this may indicate that the glucose absorbed was only partly broken down and not carried to the stage of $CO_2$ and $H_2O$.

In most specific dynamic action experiments with glucose, a rise in the respiratory quotients occurs within 2 hours after feeding. It must be borne in mind that such experiments are usually made on dogs or on man fasting 15 or 18 hours, while rabbits were the subjects in this case and were fasted *48 hours*. The exhaustion of glycogen stores after a 48 hour fast in a small animal may considerably change the immediate fate of ingested glucose.

It is in these first stages of carbohydrate decomposition that we think thyroid secretion may play its part and that is where the increased heat production results. For an explanation of this view see the discussion which follows.

DISCUSSION.

*Theory of the Mechanism of Specific Dynamic Action.*

With this new fact added to those known about specific dynamic action, that only through the agency of thyroid secretion can it be observed, a tentative concept may be formed of its mechanism.

In the disposal of some of the absorbed products of digestion, by storage or oxidation, we believe then, that thyroid secretion normally plays a part. In some of these reactions we may reasonably assume that heat is liberated. In the postabsorptive state, these same reactions in which thyroid secretion enters occur in the catabolism of foods from the body stores; the heat evolved can then be used for maintenance of body temperature. But during absorption of a meal or whenever there is what Lusk has aptly called a plethora of intermediary metabolites in a group of cells, this evolved heat may be greater than can be used for the maintenance of body temperature and for taking part in endothermic reactions (if the extra heat can be used in this way). A rise in body temperature must then result or heat be given off. The latter is the usual occurrence and the extra heat is the specific dynamic action. It is as Lusk has stated a metabolism of plethora.

This concept of the mechanism of specific dynamic action would explain why protein feeding causes the greatest specific dynamic action while fat produces little or none. In the metabolism of protein there are many more chemical changes involved than in fat metabolism, carbohydrate being intermediate in this respect. Some of these reactions are, we suppose, exothermic and more heat would be produced in disposing of the complicated protein molecule than would result from fat storage, since fat is stored in practically the same form as that in which it is eaten while most protein must be converted to glycogen or utilized directly. (We do not wish to imply that thyroid secretion does not take part in the catabolism of fat; on the contrary there is good evidence which indicates that it plays a most important rôle. Thus Marine and Lenhart (16) found that feeding hog's liver, a tissue very rich in fat, to trout fry caused thyroid hyperplasia. McCarrison (17) obtained a similar result in feeding fats to pigeons. This result has been amply confirmed and ex-

XUM

tended to many mammals by Mellanby and Mellanby (18), and by Shapiro[4] and by Seecof (19) in this laboratory.)

This theory would explain many of the confusing results found in the literature on specific dynamic action; for example, the interesting questions raised by the recent work of Rapport and Weiss (20, 21). These investigators found that proteins which contained little glycine and alanine caused as much specific dynamic action as those having 25 per cent of glycine and alanine. Lusk (22) and his school have repeatedly shown that these two amino acids cause very much more specific dynamic action than several others that they have studied;such as glutamic acid and tyrosine. But these other amino acids do cause considerable specific dynamic action and the amount produced by proteins like casein or gelatin, would depend not so much on their composition (because a considerable proportion of all protein molecules is in the form of chains that may give rise to glucose and we assume they are metabolized through the agency of thyroid secretion in much the same way as alanine and glycine) but on other factors such as the *rate* of absorption and the *maximum capacity* of the tissue to dispose of these substances as they are received. If the rate of absorption were slow enough one need never find, according to this theory, any specific dynamic action at all. On the other hand, a cell cannot be expected to increase its capacity for work indefinitely. So that even if the rate of absorption is very rapid there is a certain speed beyond which the cell cannot work.

Many other apparently anomalous results found in the literature on specific dynamic action could be explained with this theory of its mechanism but space limitations would not permit of their further discussion here.

It might be argued that instead of reacting with thyroid secretion in the cells generally, as we have postulated, the intermediary metabolites may stimulate the thyroid cells and so produce the extra heat. Such a view seems unlikely because of the very gradual rate of disappearance of specific dynamic action following thyroid removal. Moreover, after complete disappearance of specific dynamic action, feeding glucose caused the production of extra heat again if thyroid gland were fed also (see Table III).

[4] Shapiro, S., unpublished experiments.

XUM

The question naturally arises as to how these intermediary metabolites are oxidized in the completely thyroidectomized animal. That some of them, if not all, are oxidized there is no doubt. There are several possible ways in which these processes may go on and we shall mention two of them that at this time seem reasonable though it must be admitted that with our present knowledge they are entirely speculative.

1. These reactions may go on in the same way in a thyroidless animal as they do in a normal animal, but at a greatly reduced rate. The inability of such an animal to metabolize foods with the accustomed rapidity would then be the cause of decreased metabolism in myxedema and cretinism.

2. The intermediary metabolites may be oxidized in some other way. That this may occur in the oxidation of fat is indicated from the work of Schenk (23) who obtained respiratory quotients far below 70 in fasting thyroidectomized rabbits. If confirmed, such results would indicate that fat is oxidized through some other channel. It is not unlikely that both these possibilities may occur.

SUMMARY.

Complete thyroidectomy caused a gradual decrease and final disappearance of specific dynamic action after about 65 days. Restoration of specific dynamic action which had entirely disappeared following thyroid removal was accomplished by feeding thyroid gland. Incomplete thyroidectomy caused a decrease of specific dynamic action, which would be increased again by feeding thyroid and in some cases by feeding a small amount of KI.

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# THE USE OF THE QUINHYDRONE ELECTRODE FOR HYDRION CONCENTRATION DETERMINATION ON SERUM.*

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The quinhydrone electrode, as developed by Biilmann (1, 2), has proven a most convenient method for measuring the hydrogen ion concentration of solutions in many types of investigation. It is more convenient than the hydrogen electrode in ordinary solutions because special apparatus for the preparation and control of hydrogen is not necessary. In $CO_2$-containing solutions it should be especially convenient, for the greatest difficulties and the most prolific sources of error in measuring the pH of $CO_2$-containing solutions lie in the preparation and maintainence of the $CO_2$-$H_2$ mixtures.

*A priori*, several theoretical objections presented themselves to the use of the electrode for blood or serum. First, the quinhydrone electrode functions best in an acid medium, but since blood and serum are never more alkaline than pH 8 the error introduced by this factor is not important. Our experience with standard phosphate solutions is in agreement with this conclusion.

A second difficulty, that of salt effect, has been carefully studied by Sörensen, Sörensen, and Linderström-Lang (3) and by Biilmann and Lund (4). In the salt concentration of blood, about 0.15 N, this effect is also negligible.

Loss of $CO_2$ must be prevented, but this can be accomplished more easily with the quinhydrone electrode than with the hydrogen electrode (see below).

The possibility of error which is of most moment is that since

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the quinhydrone electrode is an oxidation-reduction system, whose potential is influenced by pH, other oxidation-reduction systems in the solution being investigated may change the ratio of quinone to hydroquinone to a value different from unity. The blood serum, tissue, and tissue fluids, contain complex systems of oxidizing and reducing enzymes, and it is to be expected that they will affect the added system. The velocity with which this occurs, however, may be so slow that it is possible to determine the *initial potential due to the pH*. That this is possible for serum is shown below.

Blood itself contains such a complicated system in the oxyhemoglobin-hemoglobin that it would appear almost impossible to use the quinhydrone electrode on it. This conclusion is in agreement with our results.[1]

The control technique for pH determination with the hydrogen electrode was the same as that described before by Cullen (6), with a Clark electrode as modified by Cullen (7). $CO_2$-$H_2$ mixture and Hasselbalch refill technique were used in serum equilibration with known $CO_2$ tension.

E.M.F. readings were made with a Leeds and Northrup potentiometer, lamp, and scale galvanometer. Readings were made to about 0.2 millivolt. For standardizing the system we therefore used the arrangement described by Cullen (6) in a previous paper, measuring first the E.M.F. of the chain

$$\text{Pt} \mid \text{H}_2,\ A \text{ solution} \mid \text{saturated KCl solution} \mid \text{saturated calomel electrode}$$

with a known pH of the $A$ solution.

Using the convention that the Hg of the calomel electrode is positive to the Pt of the hydrogen electrode, we have

$$\text{pH} = \frac{\text{E M.F.} - e_H}{0.0001984\ T}$$

from which $e_H$ may be calculated, and with this value of $e_H$ the same expression may be used for the calculation of pH in an

[1] Our results with blood are not in agreement with those of Corran and Lewis (5), who report normal values for normal blood obtained with the quinhydrone electrode. They did not compare their determinations with those by any other method. We are not able to explain this difference between their and our experience.

$X$ solution by measuring the E.M.F. of the chain with $X$ solution in place of $A$ solution thus

$$\text{Pt} \mid \text{H}_2,\ X \text{ solution} \mid \text{saturated KCl solution} \mid \text{saturated calomel electrode}$$

In the same way by measuring the E.M.F. of the chain

$$\text{Au} \mid \begin{matrix}\text{quinhydrone} \\ A \text{ solution}\end{matrix} \mid \text{saturated KCl solution} \mid \text{saturated calomel electrode}$$

the corresponding value $e_q$ for this type of chain was determined and, then, by measuring the E.M.F. of the chain

$$\text{Au} \mid \text{quinhydrone} \mid X \text{ solution} \mid \begin{matrix}\text{saturated} \\ \text{KCl solution}\end{matrix} \mid \begin{matrix}\text{saturated calomel} \\ \text{electrode}\end{matrix}$$

the pH of the $X$ solution was calculated from

$$\text{pH} = \frac{\text{E.M.F.} + e_q}{0.0001984\ T}$$

In every case each system was standardized against the same standard solution and the values of $e_H$ and $e_q$ *determined for the whole system*. In consequence, the accuracy of the measurements depends on the exactness of the pH value of the $A$ solution but is independent of the comparison electrode (in this case saturated calomel electrode), the liquid junction potentials, temperature coefficient, etc. The $A$ solution in our experiments was a standard phosphate, with pH close to the pH of the $X$ solution, which was in turn checked against standard HCl solution. The type of comparison electrode is incidental. (When using quinhydrone electrodes and setting up apparatus for that purpose, the quinhydrone standard electrode may be convenient (Veibel (8).)

*Electrodes.*—The large electrode vessels first used by Biilmann are not suitable for $CO_2$ solutions and also require too much material. The capillary electrode described by Biilmann and Lund was slightly modified for our purpose. We tried four different types of capillary electrodes.

Biilmann and Lund's capillary electrode (Fig. 1, *A*) is mechanically more convenient in form *B*, because it is more rigid in its connection and thus does not draw KCl up into the capillary as

*A* sometimes does. The wire (gold-plated platinum wire) is shorter and better protected. The capillary part of the tube should be at least 20 mm. long and about 1 mm. inside diameter.

*C* is *B* with a coating of De Khotinsky cement over the upper half of the wire. This is used in $CO_2$ solution so that the change of pH due to loss of $CO_2$ at the surface cannot affect the E.M.F. De Khotinsky cement, soft, is better than glass, because it is less fragile and can easily be removed or renewed in an alcohol flame.

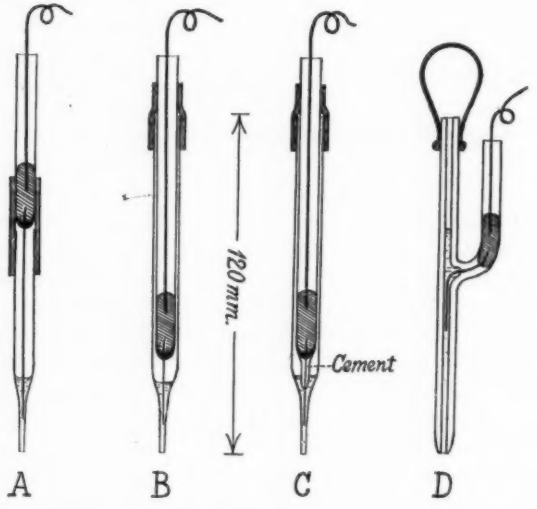


FIG. 1.

*D*, made of heavy-walled capillary glass and fitted with a rubber bulb, is the most convenient form to manipulate. It is similar to the hydrogen electrode described by Walpole (9). The surface of the solution is well above the electrode wire. It has, however, one drawback in that it does not immediately attain the temperature of the saturated KCl bridge solution.

*B* and *C* are for this reason most satisfactory for serum.

Two or more of these electrodes may be placed in the same KCl bridge and by means of a convenient throw switch can be read at 15 second intervals. In order not to disturb the electrodes it is

convenient to use light spring clips with serrated jaws soldered to flexible insulated copper wire as leads from the switch. We use the clips sold by Dennison for show-window cards. Biilmann has previously stated that gold or gold-plated electrodes may in some cases be more satisfactory than platinum, and this is also true in serum work. Because the platinum is gold-plated great care must be taken to guard it from mercury. This is done either by using platinum wire for the whole distance or if, for economy a mercury contact is used, a copper wire should be cemented in. Cotton wool soaked in hot paraffin and packed in around the copper wire is most satisfactory.

The gold-plating is best applied with a very low current (some few milliamperes) from a gold cyanide solution. The electrodes may be cleaned by immersion for several hours in concentrated sulfuric acid, arsenic-free, containing chromic acid.

The quinhydrone was prepared in the following manner: 100 gm. of ferric ammonium alum are dissolved in 300 cc. of water at about 65° and this solution is poured into a warm solution of 25 gm. of hydroquinone in 100 cc. of water. The quinhydrone precipitates in fine dark needles. (If a more dilute solution of hydroquinone is used, the crystals formed by the precipitation are bigger and do not dissolve so rapidly in water.) The mixture is cooled in ice and filtered by suction, and the precipitate then washed four or five times with cold water and dryed in the air at *room temperature*. Yield 15 to 16 gm.

## EXPERIMENTAL.

Before comparing the hydrogen and quinhydrone electrodes directly we attempted in numerous experiments to establish conditions under which we could obtain reproducible readings with the quinhydrone electrode in serum or blood. Since this was difficult and since changes in several details of technique cause error we will record our difficulties for the benefit of others.

*Serum. Use of Mineral Oil to Prevent Loss of $CO_2$.*—In obtaining serum from blood without loss of $CO_2$ it is necessary to use a cover of paraffin oil. If one has a small amount of a $CO_2$-containing solution, such as serum, covered with oil, it is difficult to add quinhydrone, for the crystals remain in the oil and dissolve in it. By first placing a generous quantity of crystals

in the bottom of a dry tube, then adding oil, one can run in the solution under the oil and by stirring with a rod dissolve some quinhydrone in a minute or two. But as shown later, this requires too much time for serum. Next we tried moistening first the quinhydrone with a small drop of water, then adding oil, and finally the solution. Sufficient quinhydrone was dissolved more quickly. The plan was to make many readings on one sample, but we were unable in this way to get satisfactory successive duplicate readings on serum.

*Time Effect.*—When quinhydrone is added to serum, the solution begins to change color in the first 15 seconds to 1 minute and in 2 or 3 minutes a definite red color develops. It is necessary to make the reading before this color develops. Addition of sodium fluoride to the serum does not delay this color change.

*Quinhydrone Added in Capillary Electrodes.*—It is possible to add the quinhydrone to the capillary electrode before addition of serum, in either of two ways: (*a*) Dry the electrode, type *C*, and shake crystals of quinhydrone into the capillary portion. Then dip the capillary point of the electrode quickly through the oil, and draw up the solution by manipulation of the inside tube. (*b*) Touch the gilded wire, moistened with either water or 0.9 per cent NaCl solution, to powdered quinhydrone and insert the wire carrying its load of crystals into the capillary. The latter method is the more convenient and the dilution is apparently negligible. However, the upper surface of the solution is exposed to air and can lose $CO_2$. An attempt was made to use technique (*a*) above, adding dry quinhydrone, then drawing up a little oil first, but the oil clings to the capillary walls and often insulates the electrode and also interferes with the solution of the quinhydrone.

The effect of the $CO_2$ loss may be eliminated by using an electrode of type *C* or *D*. Apparently the $CO_2$ loss is slight and irregular, for sometimes one cannot detect any difference when this precaution is omitted, although usually one can.

*Reproducibility of Results with Capillary Electrode.*—In reading the potential of serum the time of contact of serum with quinhydrone is controlled thus: The electrode and wire are carefully rinsed with distilled water and the water blown from the capillary. The wet wire is then touched to dry quinhydrone and

inserted into the capillary which is then plunged through the layer of oil overlying the serum in its tube. Withdrawing the inner tube, serum is drawn up into the capillary to above the lower end of the cement covering. The lower end of the wire should be at least 5 mm. from the tip of the capillary electrode tube. The quinhydrone is partially dissolved as the serum flows up. The time is noted most easily by starting a stop-watch,

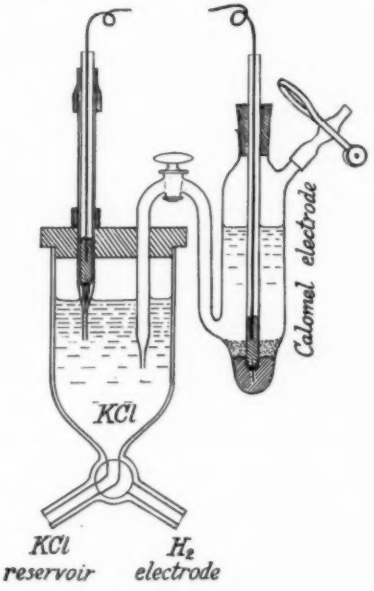


FIG. 2.

and the electrode is attached to the connecting wire of the potentiometer and then placed in the saturated KCl bridge (Fig. 2).

The first reading is finished by the half minute, then further readings are made at 1, 2, and 3 minutes. One can work with two or three electrodes at the same time. The temperature of the KCl is also read. If there is a great change between the successive readings, more than 1 to 2 millivolts, the determination is not used, but in most cases the drift for the first 2 minutes is low and the $\frac{1}{2}$ minute and minute readings are practically the

same. The minute reading is the one used for calculation, since temperature agreement with the KCl has been obtained by that time and drift has not become marked. Three or four determinations must be made and averaged.

*Control of System and Diffusion.*—The standardization of the electrode is made in the same way. When using standard solution in electrodes that are satisfactory, there is no drift due to diffusion for 10 or more minutes, so that the 1, 2, and 3 minute

TABLE I.

*Example of Reproducibility of Reading with Serum.*

| Solution. | Electrode No. | Temperature. | E.M.F. ½ min. | E.M.F. 1 min. | E.M.F. 2 min. | E.M.F. 3 min. | Calculation. |
|---|---|---|---|---|---|---|---|
| | | °C. | | | | | |
| $PO_4$ pH 7.0 | III | 18 | −0.0490 | −0.0490 | −0.0490 | −0.0490 | $e = 0.4529$* |
| | IV | 18 | −0.0490 | −0.0490 | −0.0490 | −0.0490 | |
| | | | | | | | pH at 1 min. |
| Serum. | III | 18 | −0.0025 | −0.0025 | −0.0025 | −0.0020 | 7.81 |
| | IV | 18 | −0.0028 | −0.0025 | −0.0022 | −0.0020 | 7.81 |
| | III | 18 | −0.0038 | −0.0038 | −0.0043 | | 7.78 |
| | IV | 18 | −0.0040 | −0.0045 | −0.0055 | −0.0065 | $X$† |
| | III | 18 | −0.0030 | −0.0030 | −0.0037 | | 7.80 |
| | IV | 18 | −0.0025 | −0.0028 | −0.0030 | | 7.80 |
| Average at 1 min..... | | | | | | | 7.80 |

* $7.0 = \frac{e + \text{E.M.F.}}{0.0577}$ or $e = 7.0 \times 0.0577 + 0.0490$.

† Determination rejected because of drift.

readings must agree. The electrodes should be rinsed with the standard solution once before adding the quinhydrone. It makes no difference whether quinhydrone is added to the standard solution or to the wire as described above. As we have not always been able to obtain agreement closer than 0.5 millivolt between successive determinations we always take the average of at least two and usually four determinations. We always use at least two electrodes.

A sample experiment showing the agreement between successive determinations in different electrodes is given in Table I.

The following experiment, one of several, is given as an example of the results with both electrodes on the same serum.

*Experiment to Compare Hydrogen and Quinhydrone Electrodes.*—About 300 cc. of blood were taken, as treatment, from the arm vein of a patient with hypertension. The blood ran, with stirring, into a bottle containing enough NaF to make the resulting concentration about 0.3 per cent. NaF was used to prevent glycolysis and consequent change in reaction (Evans (10)) during the time required for the two sets of determinations. 100 cc. of

TABLE II.

*Comparison of Hydrogen Electrode and Quinhydrone Electrode on the Same Serum.*

Phosphate solution pH 7.8 at 20°.

| Electrode. | E.M.F. | e |
|---|---|---|
| | *volts* | |
| Hydrogen I. | 0.7035 | 0.2503 |
| " II. | 0.7035 | 0.2503 |
| Quinhydrone III. | 0.000 | 0.4532 |
| " IV. | 0.000 | 0.4532 |

Serum equilibrated at 26 mm. $CO_2$ at 20°.
pH determinations made at 20°.

| Electrode. | E.M.F. (+ correction for $CO_2$). | | | pH* |
|---|---|---|---|---|
| | *volts* | | | |
| Hydrogen I. | 0.6856 | | | 7.50 |
| " II. | 0.6873 | | | 7.52 |
| | ½ min. | 1 min. | 2 min. | pH at 1 min. |
| Quinhydrone III. | −0.0165 | −0.0165 | −0.0175 | 7.51 |
| " IV. | −0.0170 | −0.0170 | −0.0185 | 7.50 |
| " III. | −0.0180 | −0.0175 | −0.0175 | 7.50 |
| " IV. | −0.0175 | −0.0175 | −0.0185 | 7.50 |

* A more direct method of comparing the two electrodes is to calculate the E.M.F. of the chain

Pt | $H_2$ solution | saturated KCl solution + quinhydrone | Au

Using the average values of the quindrone and hydrogen electrodes (corrected for vapor tension to 1 atmosphere of $H_2$), the potential of this chain is

Phosphate solution.....0.7037 + 0.0000 = 0.7037 volts.
Serum.....0.6871 + 0.0171 = 0.7042 "

the blood were centrifuged and the serum was removed. This is called "serum" because although the blood did not clot as a whole there were many small clots, and fibrin formed and was removed with a rod after gentle stirring from the supernatant plasma over the cells.

The serum was equilibrated in two successive tonometers—as described by Austin and coworkers (11)—at 26 mm. of $CO_2$ pressure at 20° and removed to a mercury sampling tube from which portions were run under oil into small short tubes into which it was convenient to plunge the quinhydrone capillary electrodes. The results are given in Table II. The agreement between the two types of electrodes is within 0.02 pH, which is within the error of the two methods.

*Experiments with Blood.*

Since it would be of great value to be able to use the capillary electrode directly on blood, we made many attempts to work fast enough to do this. With a small amount of quinhydrone the drift is so rapid and erratic that we could not extrapolate back to zero time. If, however, a large amount of quinhydrone is added to blood, within 10 seconds the blood becomes dark, the color of methemoglobin. Gas comes off in minute bubbles and the resulting dark mixture gives a stable potential, which does not, however, correspond to the potential of the serum from the untreated blood, but to a much more alkaline reaction. Although it is possible that this value may have a simple constant relation to the actual value, it seems so improbable that we have not investigated the possibility further. The drift in serum is not stabilized at any new value by adding large amounts of quinhydrone.

*Serum Containing Hemoglobin.*—Serum containing definite traces of hemoglobin gives drifting values and is, therefore, unsuitable for study with the quinhydrone electrode.

*Serum and Plasma.*—The reproducibility of the results with successive determinations illustrated for serum in Table I holds also for plasma. However, all our comparison experiments with both the hydrogen and quinhydrone electrodes were done on serum from blood containing NaF, as described above. Although this may have contained some fibrin we cannot now state that

plasma can be used with as much satisfaction as serum. It is, however, probable that it can be. This question will be reported on later.

## CONCLUSIONS.

Our experiments with the solutions used in this study furnish further evidence of the convenience of the quinhydrone electrode wherever secondary oxidation-reduction reactions do not interfere. The capillary electrode is an economical and convenient form of the electrode. There is, however, the same tendency for individual electrodes to vary as with the hydrogen electrodes, so that the safest procedure is to check the accuracy of the entire system against a suitable standard solution. The immediate standard solution used should preferably be one with pH near the pH of the unknown solutions.

In biological material, it is essential to determine whether the enzymatic processes interfere and, if so, whether the rate of such effect is sufficiently slow to allow determinations.

*Serum.*—In the case of serum, proper precautions as to time make it possible to use the quinhydrone electrode with apparently the same accuracy as the hydrogen electrode.

*Blood.*—Our results with whole blood indicate that the quinhydrone electrode is not suitable for use with the hemoglobin system.

We wish to thank Prof. C. Lundsgaard for putting his laboratory at the Rigshospitalet at our disposal.

## SUMMARY.

The use of the quinhydrone electrode for determining the pH of blood and blood serum has been studied.

By reading the potential immediately after addition of quinhydrone satisfactory results are obtained with serum. The details of a technique for this purpose are described.

We have not been able to use the quinhydrone electrode with whole blood.

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XUM

# THE EFFECT OF pH ON THE OXYGEN CONSUMPTION OF TISSUES.*

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## INTRODUCTION.

In simple chemical oxidation such as the oxidation of pyrogallol or of a ferrous salt, the hydrogen ion concentration plays an important rôle. In general, as in the case of pyrogallol, a slight acidity depresses oxidation, and increasing alkalinity, even to a marked degree, greatly increases the rate of oxidation. It has been assumed that in general this applied also to biological oxidation, but there have been few observations upon this subject.

Recently Büchner and Grafe (1) concluded from their experiments, using the method of Warburg, that changes of pH had little effect on the oxygen consumption of finely cut tissue suspensions. These authors state that the oxygen consumption between pH 5 and 9 is practically constant and is diminished only at either extreme, gradually at first and then more markedly. These findings are rather surprising in view of our general knowledge of oxidation, but especially in the light of other investigations.

The work of Warburg (2) and McClendon and Mitchell (3) showed that in general an increase in hydroxyl ion concentration increased the oxygen consumption of sea urchin eggs and nucleated erythrocytes.

Bunzell (4) showed that the oxidase activity of plant material is markedly sensitive to changes in pH, and that from a complete inhibition at approximately pH 3, the activity increased up to pH 7. Unfortunately the alkaline side was not investigated.

* This paper is No. 47 of a series of studies in metabolism from the Harvard Medical School and allied hospitals. The expense of this investigation has been defrayed in part by a grant from the Proctor Fund of the Harvard Medical School for the study of chronic diseases.



XUM

That the oxidation of fructose by molecular oxygen increased from pH 6.2 to 8.0 in a phosphate solution and increases in a glycocoll-NaOH buffer system up to pH 11.3 was recently shown by Warburg and Yabusoe (5).

Many of these observations are similar to the oxidation of pyrogallol in that the consumption of oxygen increases even after fairly marked alkalinity is reached. This is not always the case, as has been shown by Mathews and Walker (6) in a study of the oxidation of cysteine to cystine by molecular oxygen. These investigators found that this rate of oxidation had a fairly sharp optimum at an H ion normality of pH 8 and that slight changes in either direction greatly suppressed the rapidity of oxygen consumption. This is of special interest inasmuch as the -SH group undoubtedly plays an important part in biological oxidation. This point was further emphasized by the work of Hopkins (7) in the isolation from muscle of a thermostable compound, glutathione, with a maximum oxidative activity at approximately the pH of the blood (7.4 or slightly greater), and a marked inhibition at pH 6.8.

The optimum reaction for the oxidation of some substances may even be on the acid side of neutrality as recently has been shown by Fleisch (8). Using washed, minced muscle and citric acid, he showed that the optimum pH for the oxidation of the citric acid was about 6. At pH 7.6 the oxygen consumption was quite small in comparison.

*Methods.*

The tissues used in all the experiments were from rabbits killed instantaneously by a blow over the cervical spine. The animals were bled immediately from the carotid and jugular vessels as free of blood as possible. The tissues to be used were removed rapidly and chilled upon ice; the whole process before chilling was only of a few minutes duration. The tissues after being chilled were dissected as free as possible from connective tissue and ground up to a fine mince by means of a food chopper. Aliquot portions of the minced tissue were weighed out, about 15 cc. of Ringer's solution added, then 0.2 N acid or alkali, followed by dilution with Ringer's solution to the desired total volume.

The oxygen consumption was determined by means of the

apparatus described in the preceding paper (9). Three flasks were mounted side by side in a frame to which a shaking motion was imparted. All determinations were made in a constant temperature water bath at 37.5°C.

The pH of the tissue suspensions was determined electrometrically immediately after the rate of oxygen consumption was determined. In general, the reaction of the tissue at the completion of the experiments was slightly more acid than the average during the determination. The magnitude of the changes of tissue pH after the addition of acids or alkalies at body temperature has been discussed in previous publications (10). Only on the alkaline side of neutrality is this change at all marked, but even this shift of pH during the short duration of our experiments would not alter appreciably the general results we obtained.

TABLE I.

*Comparison of Rate of Oxygen Consumption of Heart Muscle, Skeletal Muscle, and Liver Tissue.*

| Time. | Heart muscle, 2.9 gm. + 10 cc. Ringer's solution. pH 6.71. | Skeletal muscle, 30 gm. + 30 cc. Ringer's solution. pH 6.68. | Liver tissue, 30 gm. + 30 cc. Ringer's solution. pH 6.66. |
|---|---|---|---|
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 15 | 48 | 35 | 94 |
| 50 | 109 | 98 | 185 |
| 70 | 135 | 142 | 220 |
| 90 | 165 | 162 | 250 |
| Per gm. 1st hr... | 44.2 | 4.1 | 7.2 |

RESULTS.

A comparison of the rates of oxygen consumption of heart and skeletal muscle and liver tissue from the same rabbit is presented in Table I.

The rate of oxygen consumption of minced heart muscle suspended in Ringer's solution was extremely great, 44.2 c. mm. per gm. the 1st hour, while that of skeletal muscle was only about one-tenth as great, 4.1 c. mm. per gm. the 1st hour. The oxygen consumption of liver tissue 7.2 c. mm. per gm. the 1st hour, was intermediate between that of heart and skeletal muscle. No attempt was made to adjust the reaction of the tissue suspensions

TABLE II.

*Rates of Oxygen Consumption of Liver Tissue at Various pH Values.*

Experiment 1.

| Time. | 20 gm. liver tissue + 0.2 N NaOH, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 5 cc. NaOH. pH 7.27. | 10 cc. NaOH. pH 8.96. | 15 cc. NaOH. pH 9.65. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 15 | 160 | 90 | 35 |
| 30 | 220 | 143 | 55 |
| 50 | 285 | 186 | 70 |
| 83 | 345 | 225 | 92 |
| Per gm. 1st hr... | 15.5 | 10.3 | 4.0 |

Experiment 2.

| Time. | 20 gm. liver tissue + 0.2 N NaOH, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 3 cc. NaOH. pH 6.90. | 4 cc. NaOH. pH 7.05. | 5 cc. NaOH. pH 7.30. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 15 | 50 | 42 | 72 |
| 30 | 90 | 120 | 160 |
| 60 | 162 | 210 | 222 |
| 90 | 220 | 285 | 298 |
| Per gm. 1st hr... | 8.1 | 10.5 | 11.1 |

Experiment 3.

| Time. | 20 gm. liver tissue + 0.2 N NaOH, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 4 cc. NaOH. pH 7.18. | 6 cc. NaOH. pH 7.56. | 7 cc. NaOH. pH 7.83. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 22 | 130 | 121 | 98 |
| 37 | 190 | 176 | 136 |
| 50 | 220 | 208 | 195 |
| 60 | 242 | 230 | 220 |
| 84 | 270 | 250 | 237 |
| Per gm. 1st hr... | 12.1 | 11.5 | 11.0 |

XUM

TABLE II—*Concluded.*

Experiment 4.

| Time. | 15 gm. liver tissue + 0.2 N NaOH, made up to 30 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 2.25 cc. NaOH. pH 7.08. | 3.00 cc. NaOH. pH 7.46. | 3.75 cc. NaOH. pH 7.75. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 7 | 10 | 32 | 20 |
| 15 | 32 | 80 | 50 |
| 25 | 65 | 100 | 85 |
| 34 | 92 | 142 | 112 |
| 50 | 160 | 200 | 173 |
| 60 | 190 | 214 | 195 |
| Per gm. 1st hr... | 12.6 | 14.3 | 12.7 |

Experiment 5.

| Time. | 10 gm. liver tissue + 0.2 N HCl, made up to 25 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 3.5 cc. HCl. pH 5.5. | 1.5 cc. HCl. pH 5.87. | 0.0 cc. HCl. pH 6.66. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 18 | 16 | 37 | 45 |
| 23 | 19 | 48 | 54 |
| 37 | 27 | 65 | 75 |
| 60 | 35 | 72 | 90 |
| Per gm. 1st hr... | 3.5 | 7.2 | 9.0 |

in this experiment and the comparisons are based upon the pH values that are obtained when tissues are removed from the animal organism; that is, approximately 6.4 to 6.7 (10). The rate of oxygen consumption in all tissues studied gradually decreased with time at 37.5°C. This was especially apparent during the 2nd hour of the experiment.

Since it was impossible to get more than a few gm. of heart muscle from a rabbit, all further studies were made upon skeletal muscle and liver tissue.

Table II presents the data of a series of experiments showing the effects of change in reaction on the rate of oxygen consumption of liver tissue. In the first four experiments, various amounts of

XUM

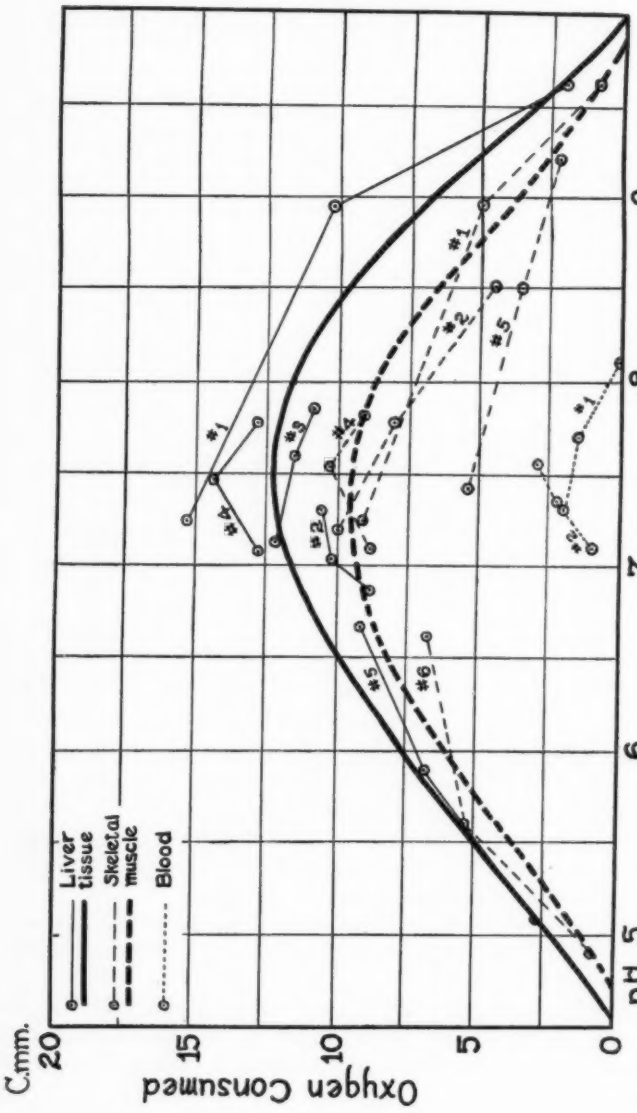


FIG. 1. Showing the c. mm. of oxygen consumed by 1 gm. of tissue or 1 cc. of blood during the 1st hour at various pH values.

TABLE III.

*Rates of Oxygen Consumption of Skeletal Muscle Tissue at Various pH Values.*

Experiment 1.

| Time. | 20 gm. muscle tissue + 0.2 N NaOH, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 5 cc. NaOH. pH 7.29. | 10 cc. NaOH. pH 8.95. | 15 cc. NaOH. pH 9.64. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 15 | 72 | 30 | 5 |
| 45 | 135 | 80 | 15 |
| 55 | 162 | 94 | 28 |
| 75 | 188 | 115 | 40 |
| Per gm. 1st hr... | 8.4 | 5.0 | 1.5 |

Experiment 2.

| Time. | 20 gm. muscle tissue + 0.2 N NaOH, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 5 cc. NaOH. pH 7.22. | 7.5 cc. NaOH. pH 7.78. | 10 cc. NaOH. pH 8.50. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 20 | 52 | 30 | 10 |
| 33 | 115 | 52 | 30 |
| 43 | 142 | 110 | 70 |
| 50 | 170 | 1 5 | 85 |
| 60 | 200 | 145 | 93 |
| Per gm. 1st hr... | 10.0 | 7.7 | 4.6 |

Experiment 3. Same rabbit as in Experiment 2 but muscle kept on ice for 24 hrs.

| Time. | 20 gm. muscle tissue + 0.2 N NaOH, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 6 cc. NaOH. pH 7.48. | 8 cc. NaOH. pH 8.11. | 12 cc. NaOH. pH 9.22. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 15 | 30 | 0 | 0 |
| 45 | 69 | 20 | 0 |
| 90 | 110 | 35 | 22 |
| Per gm. 1st hr... | 3.7 | 1.5 | 0.7 |

TABLE III—*Concluded.*

Experiment 4.

| Time. | 20 gm. muscle tissue + 0.2 N NaOH, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 4 cc. NaOH. pH 7.15. | 6 cc. NaOH. pH 7.52. | 7 cc. NaOH. pH 7.80. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 13 | 45 | 55 | 50 |
| 20 | 70 | 105 | 76 |
| 40 | 117 | 175 | 135 |
| 52 | 145 | 198 | 165 |
| 68 | 170 | 215 | 185 |
| Per gm. 1st hr... | 8.3 | 10.4 | 8.9 |

Experiment 5.

| Time. | 20 gm. muscle tissue + 0.2 N NaOH, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 5 cc. NaOH. pH 7.46. | 7.5 cc. NaOH. pH 8.50. | 10 cc. NaOH. pH 9.25. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 9 | 50 | 8 | 0 |
| 30 | 97 | 18 | 15 |
| 48 | 117 | 40 | 30 |
| 61 | 122 | 60 | 48 |
| 80 | 140 | 70 | 69 |
| 100 | 160 | 83 | 78 |
| Per gm. 1st hr... | 6.0 | 3.0 | 2.4 |

Experiment 6.

| Time. | 20 gm. muscle tissue + 0.2 N HCl, made up to 40 cc. with Ringer's solution. | | |
|---|---|---|---|
| | 0.0 cc. HCl. pH 6.61. | 3.0 cc. HCl. pH 5.58. | 7.0 cc. HCl. pH 4.48. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 27 | 55 | 47 | 5 |
| 47 | 112 | 72 | 12 |
| 60 | 142 | 112 | 15 |
| Per gm. 1st hr... | 7.1 | 5.6 | 0.8 |

0.2 N NaOH were added so as to cover the pH range from 6.9 to 9.65. In Experiment 5, 0.2 N HCl was added to the liver suspension so as to extend the pH range further toward the acid side. At the extreme acid and alkaline limits, pH 5.5 and 9.65, oxygen consumption was greatly depressed. The maximum rates of

TABLE IV.

*Rates of Oxygen Consumption of Whole Blood, Freshly Drawn from Normal Individuals and Adjusted to Various pH Values.*

Experiment 1. Blood from R. J. R.

| Time. | 15 cc. blood + 0.2 N HCl and made up to 30 cc. with Ringer's solution and aerated free of $CO_2$. | | |
|---|---|---|---|
| | 0.0 cc. HCl. pH 8.06. | 1.5 cc. HCl. pH 7.71. | 3.0 cc. HCl. pH 7.32. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 15 | 0 | 0 | 10 |
| 30 | 0 | 14 | 22 |
| 60 | 0 | 25 | 30 |
| Per cc. 1st hr.... | 0 | 1.7 | 2.0 |

Experiment 2. Blood from J. C.

| Time. | 30 cc. blood + 0.2 N HCl and made up to 60 cc. with Ringer's solution and aerated free of $CO_2$. | | |
|---|---|---|---|
| | 4 cc. HCl. pH 7.51. | 6 cc. HCl. pH 6.34. | 7 cc. HCl. pH 7.12. |
| *min.* | *c. mm. oxygen* | *c. mm. oxygen* | *c. mm. oxygen* |
| 10 | 20 | 17 | 15 |
| 15 | 33 | 26 | 22 |
| 37 | 72 | 55 | 38 |
| 60 | 90 | 64 | 50 |
| 120 | 165 | 105 | 92 |
| Per cc. 1st hr.... | 3.0 | 2.1 | 1.3 |

oxygen consumption of liver tissue were obtained in those experiments in which the pH was adjusted approximately 7.3 to 7.6. In Fig. 1 (1) each experiment on liver tissue is represented by the light continuous lines and the general effect of pH on the oxygen consumption is approximated by the heavy curve.

Table III gives the data for similar experiments using skeletal

XUM

muscle. Experiment 3 is a duplicate of Experiment 2, except that the minced muscle was kept on ice for 24 hours. Unfortunately the pH values were not quite the same, but it is apparent that the muscle tissue had lost a good deal of its power to consume oxygen. For the sake of comparison with liver tissue each individual experiment on skeletal muscle together with an ideal curve is represented by broken lines in Fig. 1. It will be noted that for any given pH, muscle tissue consumes on the average less oxygen than liver tissue, but that the general effect of change of pH is approximately the same for the two tissues.

The experiments both on muscle and liver tissue from different animals but otherwise under identical conditions, showed a considerable variation in ability to utilize oxygen. It is difficult to tell whether this variation was due to inherent differences in the vital tissues, such as food content or enzymatic conditions, or to subsequent changes either during or after death of the animals. The latter probably is more reasonable, for such factors as shock during death and differences in the completeness of the removal of the blood from the tissues could easily influence the rapidity of oxidation.

Table IV gives the data of two experiments on the effect of changes in pH of whole blood from normal individuals upon its ability to utilize oxygen. The experiments are plotted in Fig. 1 (dotted lines) and show the small consumption of oxygen as compared with the other tissues. The effect of changes of pH on the oxygen metabolism of the blood was roughly comparable to that of liver and muscle tissue, but enough points were not available to establish clearly a complete curve.

## DISCUSSION.

That tissues continue to utilize oxygen for some time after their removal from the body has been proved abundantly and known for a long time. Careful quantitative studies under controlled conditions have, however, been few. An interesting question is the relationship of oxygen utilization in minced tissue suspensions to that of the intact organ within the animal. It is, of course, apparent that the two conditions are not comparable and that widely divergent figures should be expected. In the first place,

XUM

in the mincing of the tissues, structures are destroyed. The character of tissues and cells plays an important part in intracellular reactions. This is well known and is thoroughly discussed and reviewed by Lillie (11). In the second place divergent results might be expected because of a different physiological environment, the absence of nervous control and tonus, limitation of food supply, and, perhaps of greatest importance, the change in oxygen tension. Normally the oxygen tension in tissues is very low, perhaps only a few mm., while in our experiments with atmospheric air, it, of course, was very much greater. However, our experiments with minced tissue in suspension agree in general with those on the intact organs by earlier workers. In particular the experiments of Barcroft and his associates (12) and Evans (13) are of interest for they demonstrated that the intact cat's liver used from 3.0 to 10.8 c. mm. of oxygen per gm. per hour while fasting and from 14.4 to 30.0 c. mm. per hour when the animal was previously fed. The average oxygen consumption of minced liver in our experiments at pH 7.4 was 10 to 15 c. mm. per gm. per hour, the animals having had no food for about 15 hours. In regard to the heart no comparisons can be made, for the heart-lung preparation studies of Evans were made on the contracting heart. The normally beating heart, according to Evans, consumed from 25.8 to 51.0 c.mm. of oxygen per hour. In our experiments at a pH of 6.7, the unadjusted pH of minced heart muscle, the oxygen consumption was 44.2 c.mm. per hour. This high rate of utilization is probably due to shreds of cardiac musculature still maintaining in part their property of rhythmic automaticity together with the increased oxidation due to the increased oxygen tension.

Our experiments also showed that liver tissue consumes more oxygen than skeletal muscle tissue, this probably is due to the greater storage of food together with the diversified metabolic activities of the former organ.

In respect to the effect of pH on the oxygen consumption of tissues, our results differ from those of Büchner and Grafe (1) who found little effect between pH 5 and 9. In all our experiments, including those on blood, the maximum rate of oxygen consumption was in the vicinity of the normal reaction of the blood, pH 7.4 to 7.5. These changes can best be seen from

Fig. 1. This optimum reaction is in harmony with the findings of Hopkins (7) in regard to glutathione, a substance that undoubtedly plays an important rôle in tissue oxidation.

It must be borne in mind that the depression of the oxidation on the alkaline side may not necessarily be due to an increase of pH within the cell. Although NaOH was added, in the presence of the $CO_2$ of the tissue this was changed to $NaHCO_3$, thereby increasing the amount of $CO_2$ present and thus probably increasing the diffusion of $CO_2$ into the cell. This view of increased cellular acidity resulting from the administration of $NaHCO_3$ was first advanced by Gesell (14).

It is also possible that the increased alkalinity does not directly effect the oxidative process in the tissue, but that the consumption of oxygen is hindered by altering the physical condition of the suspension. That such a physical change results upon addition of alkali to a tissue suspension is well known. In our experiments is was apparent because the suspensions upon addition of alkali appeared more viscid. However, it is reasonable to assume from the data given, that variation in either direction from the normal pH of the tissue of the living organism results in a depression of the oxidative process.

## CONCLUSIONS.

The rate of oxygen consumption of the minced tissue suspensions studied is a function of the hydrogen ion concentration. The optimum pH was found to be in the vicinity of the normal reaction of the blood, pH 7.4 to 7.5. At pH values less than 4.5 and greater than 10, oxygen consumption is nearly completely depressed.

Different tissues are affected in a similar manner by changes in pH, but at the same pH, various tissues have different rates of oxidative activity. Heart muscle is most active, liver tissue much less, and skeletal muscle still less.

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XUM

# ON THE AGING OF FERROUS HYDROXIDE AND FERROUS CARBONATE.

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Interest in the aging of metal hydroxide has increased greatly during the past few years and it is due, no doubt, mainly to the fact that certain metal hydroxides have become useful for the isolation of enzymes and vitamins. The selective adsorption properties of these hydroxides, aluminum hydroxide for example, make them suitable for these purposes. Their surface forces make it possible to separate unstable organic groups or compounds which otherwise would not be obtained at all, or only with extreme difficulty. But the theoretical interest in the aging phenomenon has also become more intense, since it is possible by modern methods, especially by that of X-ray spectroscopy, to follow the changes taking place in the hydroxides with time, and thus to come to certain conclusions as to the nature of the adsorption process. In this connection we have in mind particularly the recent work of Fricke and Wever (1) on aluminum hydroxide.

Our own work is concerned entirely with ferrous hydroxide and ferrous carbonate. These substances are widely distributed in nature, and almost certainly play a very large part in many biological processes. Our experiments were not originally planned with the purpose of getting data on the aging problem. But they led us to believe and finally convinced us that ferrous hydroxide and ferrous carbonate, at the instant of precipitation, possess properties which disappear very rapidly with time.

Since it was known that ferrous hydroxide and ferrous carbonate are exceedingly autoxidizable and, as a result of the absorption of oxygen, form a series of compounds it was necessary to adopt two distinct types of experiments in our study. The experiments had to be performed in: (1) the entire absence of oxygen:



XUM

and (2) the presence of air or oxygen. Much experimental material exists concerning the behavior of ferrous hydroxide and ferrous carbonate when oxygen has access to them, but as far as we are aware, very little is known about them when they are precipitated under conditions which exclude oxygen.

We shall first discuss, briefly, the work that Just and Günzburg (2) have done in this field. They made an exact study of the solubilities of metallic iron and ferrous carbonate. They found, among other things, that the solubility of fresh ferrous carbonate is higher than that which has been aged, and that fresh ferrous carbonate has the property of increasing the supersaturation of ferrous carbonate, whereas aged ferrous carbonate cannot do so. They concluded from this fact that "the ions of the ferrous bicarbonate must be different in their make-up than those of the aged ferrous carbonate. A further explanation of the whole question seems to demand additional experimental investigation of ferrous carbonate in its solid form."

In contrast to our very sparse information as to the chemical and physical behavior of ferrous hydroxide and ferrous carbonate in the absence of air or oxygen, we have numerous publications on the action of ferrous salts when they are in contact with oxygen. We have published some of the results obtained in such work (3), and will here limit ourselves to a brief review of the action of oxygen on ferrous hydroxide and ferrous carbonate in as far as it concerns the formation of magnetic oxides.

If ferrous hydroxide is oxidized under limited access of air, it can be shown in various ways that the oxidation goes mainly to the stage $Fe_3O_4 \cdot xH_2O$. In only a few cases does the oxidation proceed completely according to the scheme

$$\ddot{Fe} + \oplus = \dddot{Fe}$$

In by far the most cases the oxidation goes through numerous intermediate stages and we are very far from a thorough understanding of the mechanism of the oxidation. Nature itself demonstrates to us through the large number of magnetic and nonmagnetic ferro-ferric and ferric oxides, with their great variety of color shades, that these oxidation processes must be exceedingly complex. Then, if the processes are further complicated by the

presence of other metals than iron and by the addition of organic compounds, the possible compounds and processes become very numerous indeed.

As a working scheme we have adopted a formulation according to the theory of Werner which will be briefly described as follows:

When oxygen has access to ferrous hydroxide, the following process very probably takes place.

$$\left[Fe(OH_2)_6\right](OH)_2 + O_2 \rightarrow$$

$$\left[\begin{matrix} O_2 \\ Fe(OH_2)_5 \end{matrix}\right](OH)_2 + 2Fe\begin{matrix} OH \\ OH \end{matrix} = \left[Fe\left(\begin{matrix} O_2 \\ OH \\ OH \\ OH_2 \end{matrix} Fe\right)_2\right](OH)_2$$

I. II.

From this graphical representation it is seen that at first an oxygen molecule reacts with ferrous hydroxide and that the resulting peroxo compound (I) can combine with the surrounding, but as yet unchanged, ferrous hydroxide molecules, and thus forms what may be called a polynuclear complex salt (II). This complex salt has, however, a very short life, and through the splitting off of the coordinatively bound oxygen there is developed a very labile, ferro-ferric compound in which the proportion of ferrous to ferric iron is 2 to 1. Hauser (4) has synthesized this weakly paramagnetic oxide $Fe_2O_3 \cdot 4FeO \cdot xH_2O$ from ferro-ferric ammonium carbonate, and has found that, on further oxidation with molecular oxygen, it is converted into the magnetite $FeO \cdot Fe_2O_3$. Actually the proportion of ferrous to ferric iron is less than this formula indicates. If one passes oxygen over this magnetite at a temperature of 200°C. it is still further oxidized, as we have found into the ferric oxide, $Fe_2O_3$, and becomes even more ferromagnetic. The reason why we are concerned with the magnetic properties of the oxides of iron is made clear in our previous paper (3). *From the experiments described there it became particularly clear that the properties of the oxides were really determined by conditions existing at the moment of precipitation of the hydroxide from which they were derived.* We could also conclude that the reduction of potassium nitrate

with oxygen and ferrous hydroxide is a molecular reaction. As far as the present paper is concerned the important result was that the reduction of nitrate to nitrite was *extremely rapid, and that on the aging of the precipitated hydroxide of only a few seconds the reduction of the nitrate was decreased more than half.*

The foregoing observations relate to the things observed when working in the presence of air or oxygen. But we have also observed that ferrous hydroxide is subject to an aging process in the *absence* of oxygen, and the phenomena are in complete accordance with the observations and conclusions of Just and Günzburg, which have already been discussed.

To secure absence of oxygen from our solutions of ferrous sulfate and sodium hydroxide, from which the ferrous hydroxide was precipitated, it was necessary to boil each solution separately and to cover them with atmospheres of pure hydrogen previous to the mixing. This was conveniently done with the apparatus shown in Fig. 1.

We usually had the lower bulb half full of sodium hydroxide solution and the upper bulb half full of a solution of ferrous sulfate. On boiling, the air and steam escape through the barometer tubes dipping into wells of mercury. This arrangement of barometer tubes also acts as a safety valve against too high pressures while boiling. As the bulbs cool off, the pressures decrease and hydrogen is drawn in until the solutions have cooled off to room temperature. When cool, the ferrous sulfate in the upper bulb is run into the lower one, and becomes mixed with the solution of sodium hydroxide. A snow-white ferrous hydroxide is formed which remains white, even on standing several days, and apparently does not change in any way. *Nor does it decompose water or change in any way in general appearance, even if the mixture containing the precipitate is boiled for several hours.* However, if only a trace of air is admitted, the white flocculent precipitate changes to a green color which, in turn, becomes black if air is admitted in larger quantities.

If we repeat the experiment, but with the difference that the solution in the lower bulb contains potassium nitrate, in addition to sodium hydroxide, the results are exactly the same. A white flocculent precipitate is formed which, even on boiling for a long time, remains the same in color. It does not matter how much

or how little nitrate is present. If oxygen is kept away, there is no change in the nitrate at room temperature, nor at the boiling temperature. The fact that the ferrous hydroxide remains white and does not change from white to green or black is con-

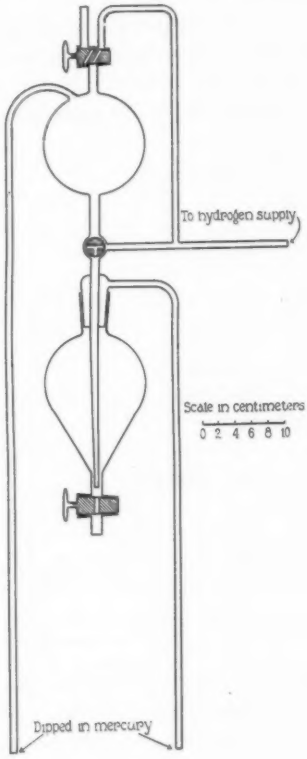



FIG. 1.

vincing evidence that the potassium nitrate has not been changed. But, in order to make doubly sure, direct tests for the presence of nitrite or ammonia in the solution, which had contained the precipitate for several days, were made. There were no nitrites or ammonia, and hence no reduction product.

The results are entirely different if, instead of potassium nitrate, potassium nitrite is used. Even in the complete absence of oxygen, the nitrite reacts with ferrous hydroxide and forms a deep green complex compound which, on decomposition, yields ammonia as a reduction product.

Before passing to a detailed consideration of the aging process, it is well to describe some quantitative experiments on the reduction of nitrates with oxygen and ferrous hydroxide. The usual procedure was as follows, using the apparatus shown in Fig. 2.

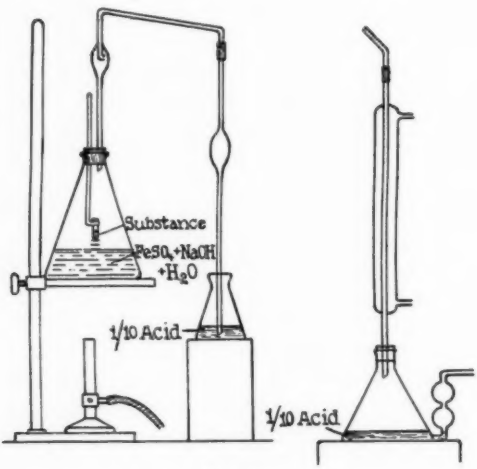



FIG. 2.

In the flask were put 400 cc. of distilled water, in which had been dissolved known quantities of sodium hydroxide and of the potassium nitrate to be reduced.[1] Then 100 cc. of a solution of ferrous sulfate were added. The solutions were thoroughly mixed by shaking. The mixture was then distilled until the

[1] With the simple apparatus shown in Fig. 2, we can also show that potassium nitrate is not reduced in the *absence* of oxygen. The oxygen being first excluded by boiling the mixture of ferrous sulfate and sodium hydroxide, the potassium nitrate is added afterwards by means of the small test-tube suspended from the stopper and indicated in the drawing as "substance."

TABLE I.*

*Reduction of Alkali Nitrate by Ferrous Hydroxide.*

Amounts used: 0.1702 gm. of $NaNO_3$ in 500 cc. of water. Complete reduction would give 0.0340 gm. of $NH_3$.

| Experiment No. | $FeSO_4$ | NaOH | Equivalent. | | Concentration of free NaOH. | $NH_3$ formed. | |
|---|---|---|---|---|---|---|---|
| | | | $Fe(OH)_2$ | Free NaOH. | | Amount. | Theoretical amount. |
| | *gm.* | *gm.* | | | *per cent* | *gm.* | *per cent* |
| 1 | 4.8 | 2.6 | 2 | 0 (neutral). | 0 (neutral). | 0.0340 | 100 |
| 2 | 4.8 | 3.2 | 2 | 0.5 | 0.12 | 0.02244 | 66 |
| 3 | 4.8 | 3.9 | 2 | 1 | 0.26 | 0.01309 | 38.5 |
| 4 | 4.8 | 5.2 | 2 | 2 | 0.52 | 0.00952 | 28 |
| 5 | 4.8 | 6.5 | 2 | 3 | 0.78 | 0.00544 | 16 |
| 6 | 4.8 | 7.8 | 2 | 4 | 1.04 | 0.00476 | 14 |
| 7 | 4.8 | 9.1 | 2 | 5 | 1.30 | 0.00425 | 12.5 |
| 8 | 4.8 | 18.0 | 2 | 11.8 | 3.08 | 0.00344 | 10 |
| 9 | 4.8 | 20.0 | 2 | 12 | 3.10 | 0.00323 | 9.5 |
| 10 | 4.8 | 23.2 | 2 | 15.8 | 4.14 | 0.00306 | 9 |
| 11 | 4.8 | 27.0 | 2 | 18.7 | 4.88 | 0.00289 | 8.5 |
| 12 | 4.8 | 35.1 | 2 | 25 | 6.50 | 0.00187 | 5.5 |
| 13 | 4.8 | 36.0 | 2 | 25.7 | 6.68 | 0.00153 | 4.5 |

* From Baudisch, O., and Mayer, P., *Biochem. Z.*, 1920, cvii, 1.

TABLE II.*

*Reduction of Alkali Nitrate by Ferrous Hydroxide in the Presence and Absence of Oxygen.*

0.1702 gm. of $NaNO_3$, 4.8 gm. of $FeSO_4$, 500 cc. of $H_2O$.

| Experiment No. | NaOH | Equivalent. | | Amount of nitrate reduced. | |
|---|---|---|---|---|---|
| | | Free. | NaOH | With oxygen. | Without oxygen. |
| | *gm.* | | *per cent* | *per cent* | *per cent* |
| 14 | 2.6 | 0 (neutral). | 0 | 100 | 0 |
| 15 | 4.0 | 1 | 0.28 | 38.5 | 0 |
| 16 | 9.1 | 5 | 1.38 | 12.5 | 0 |
| 17 | 20.0 | 12 | 3.1 | 9.5 | 0 |

* From Baudisch, O., and Mayer, P., *Biochem. Z.*, 1920, cvii, 1.

Nessler reagent no longer showed a yellow color, the distillation being usually complete when 150 cc. of liquid had been boiled off. The results are exhibited in Tables I and II and in Fig. 3.

From the tables and the figure it is seen that, when the nitrate is reduced by ferrous hydroxide, in the presence of air, through potassium nitrite, to ammonia, the behavior is quantitatively regular. With increased concentration of free alkali in the solution, that is, with increasing amount of alkali not used by the ferrous sulfate, the yield of ammonia and hence the amount of nitrate which is reduced falls off in a manner entirely quantitative. If the same experiment is carried out in such a way as to exclude air or oxygen, the nitrate remains absolutely unchanged. We regard the action of the alkali as being that of an "Anlagerung" of NaOH to the molecules of ferrous hydroxide, similar

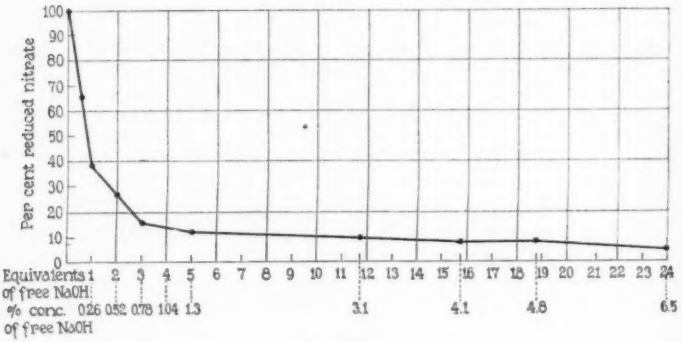



FIG. 3.

to that which Miolatti and Bellucci (5) have shown to take place between potassium hydroxide and platinum hydroxide. Their results may be expressed by the reaction

$$Pt(OH)_4 + 2KOH \rightarrow (OH)_4 Pt \begin{matrix} OHK \\ OHK \end{matrix} \rightarrow \left[Pt(OH)_6\right]K_2$$

What may here be considered as the "blocking" action of the alkali is in complete harmony with our previous explanation of the mechanism of nitrate reduction. We are dealing with a competition between the oxygen and the alkali molecules for the unsaturated ferrous hydroxide molecules. If the solution is neutral all of the ferrous hydroxide molecules can combine with the oxygen dissolved in water and form the peroxide

$$[FeO_2](OH)_2$$

Each peroxide molecule reacts with a nitrate molecule and on the splitting off of an oxygen atom from the latter a nitrite is formed. On the other hand, if free alkali is present in the solution, ferrous hydroxide presumably combines with this according to the reaction

$$Fe(OH)_2 + NaOH \rightarrow [Fe(OH)_3]Na$$

and the ferrous hydroxide is no longer available for the reduction of nitrate.

It was found that the oxygen which becomes coordinatively bound to the ferrous hydroxide can be used not only for the reduction of nitrate, but it is also capable of oxidizing certain inorganic and organic compounds. A complete discussion will be made the subject of another paper. We shall only point out here that our view of the special properties of ferrous hydroxide-peroxide is completely valid in this case. As more and more molecules of the substance to be oxidized, lactic acid, for example, combine with the nascent ferrous hydroxide-peroxide molecules, there is a corresponding increase in the yield of pyruvic acid.

From all of the foregoing it can be seen that the study of the reduction of nitrate has enabled us to gain a considerable insight into the mechanism of this peculiar action of ferrous hydroxide and oxygen. But the study has, in addition, provided us with a foothold from which the investigation of the aging of the ferrous hydroxide or carbonate may be carried out in the absence of oxygen.

If ferrous hydroxide is precipitated in the absence of oxygen, the white voluminous precipitate formed in the lower bulb of Fig. 1 settles gradually to the bottom with no apparent change in its properties. If the experiment is again carried out with the difference that potassium nitrate is also present during the precipitation, no visible change in the precipitate with time is observed. There is, then, no obvious reason for believing that any change has taken place in the hydroxide, even after it has become several hours, or even days, old. If air is admitted through the slender tube extending down into the lower bulb, and allowed to bubble through the mixture, the ferrous hydroxide changes through several intermediate stages into the red ferric hydroxide,

$Fe(OH)_3$, so that there seems to be no difference between fresh and aged ferrous hydroxide, as far as the property of absorbing oxygen is concerned. This may, indeed, be the reason that the rapid aging of ferrous hydroxide has heretofore escaped detection. However, although the aged hydroxide or ferrous carbonate retains its marked property of being autoxidizable, it *has lost completely the property of being able to reduce nitrate or to oxidize organic compounds such as uracil and lactic acid.*

Several experiments have been made to illustrate this.

*Experiment 1.*—If ferrous carbonate (10 gm. of $Na_2CO_3$ and 3 gm. of $FeSO_4$) is precipitated in the presence of 0.1 gm. of sodium nitrate in a sealed tube, preventing entrance of air, and keeping at room temperature, there is formed a snow-white product which does not change in appearance after standing for over 6 hours. If the tube is then opened to the air, and is shaken, the white ferrous carbonate takes on a deep blue-green color in a minute or two, as a result of autoxidation. In spite of continued shaking, and addition of oxygen, the nitrate is not reduced and not a trace of nitrite or ammonia is found in the filtrate.

Ferrous hydroxide was tested in a similar manner, with similar results, only that the nitrate was added to the mixture after the ferrous hydroxide had been aged.

*Experiment 2.*—1.3 gm. of sodium hydroxide were dissolved in 500 cc. of water, and the solution was boiled to drive off oxygen. Taking care that no air entered, a solution containing 4.4 gm. of ferrous sulfate was added and the precipitate was left in the sealed flask to age for 8 hours. Then the flask was opened, nitrate added, and the mixture shaken with air. The white precipitate was still autoxidizable, turning first dark green, and finally red as the iron became oxidized. The nitrate, however, remained unchanged, the tests for nitrite and ammonia being all negative.

These experiments prove that ferrous carbonate and ferrous hydroxide, *when aged out of contact with oxygen, lose the property of reducing nitrates to nitrites, althouth they retain their ability to absorb oxygen from the air and can be oxidized to ferric compounds.*

This fact becomes even more striking if an attempt is made to use the ferrous hydroxide or ferrous carbonate, which has been aged for a few hours, for the purpose of oxidizing certain organic compounds, let us say, uracil or lactic acid. These organic compounds can, as has been mentioned, easily be oxidized by *freshly* precipitated ferrous hydroxide or ferrous carbonate if air has access. On the other hand, if the ferrous hydroxide or ferrous

XUM

carbonate be left to age for a few hours, they remain autoxidizable, but the molecular oxygen then absorbed can no longer oxidize the uracil or the lactic acid which may be present.

The following experiments sustain this conclusion.

*Experiment 3.*—0.015 gm. of uracil and 5 gm. of sodium bicarbonate are dissolved in 400 cc. of water. This may be called Solution *A*. To this is added a solution containing 5 gm. of ferrous sulfate and the mixture is shaken with air until all of the iron is oxidized to Fe $(OH)_3$. The filtered solution shows every reaction characteristic of oxidized uracil.

*Experiment 4.*—Experiment 3 is repeated with the difference that Solution *A* is first boiled to drive off oxygen. Ferrous carbonate is added to the cooled Solution *A*, care being taken to keep air away, and the mixture is allowed to stand for 4 hours. Then the flask is opened and shaken with air until the iron is oxidized to Fe $(OH)_3$. The filtrate obtained in this experiment showed no oxidized uracil. It contained only unchanged uracil. Although the precipitation of ferrous bicarbonate took place in the presence of uracil, there was no oxidation on the subsequent admission of oxygen. The ferrous bicarbonate had been aged, and was ineffective as an aid to the oxidation.

Similar results were obtained on the oxidation of lactic acid to pyruvic acid. Here, too, it is true that it is only the *freshly* precipitated ferrous bicarbonate which is able to bring about oxidation when oxygen is admitted. After the bicarbonate has aged for a few hours, it is still, indeed, autoxidizable, but it is not able to aid the oxidation of lactic acid.

From the experimental results we have here described, and from our previous work, there appears a whole series of facts which will now be briefly analyzed.

Freshly precipitated ferrous hydroxide alone does not react with potassium nitrate nor with the alkali salts of, say, lactic acid or succinic acid, nor does molecular oxygen alone react with these same compounds. However, if one gives both of the substances, ferrous hydroxide and molecular oxygen, the opportunity to react with each other, there is formed a chemical compound having an extraordinarily great reactive tendency which can change the potassium nitrate, if simultaneously present, to potassium nitrite. This strongly reactive ferrous hydroxide-peroxide can also oxidize lactic acid into pyruvic acid. Both of these reactions, the oxidation, as well as the reduction, are a direct function of the oxygen dissolved in the water which contains the

XUM

reacting substances. This molecular oxygen in solution is activated as a *molecule*. If it is still a molecule when it performs the oxidation, or if it is first split up into the atoms, we are not able to decide with our present experimental material. But the fact remains that under the given conditions, namely attachment to the iron atom, the oxygen has gained a markedly great chemical activity. This activity is so great that even in the strongly reducing medium formed by an excess of unchanged ferrous hydroxide molecules it is able to oxidize such substances as lactic acid or succinic acid, which are ordinarily so difficult to oxidize.

This great oxidizing ability belongs, however, only to the oxygen which has been activated by the *freshly precipitated* ferrous hydroxide or ferrous bicarbonate. If the freshly precipitated ferrous hydroxide or ferrous bicarbonate is rapidly centrifuged out and then used for autoxidation in the presence of the substances mentioned, there is not the slightest trace of any chemical change in them. The coordinatively linked oxygen attached to the iron atom of the ferrous hydroxide which has been aged for so short a time possesses no longer such a high oxidation potential. It is only able to oxidize those substances having very labile hydrogens, such as they are in para-phenylenediamine, or in benzidine. This oxygen can also no longer reduce potassium nitrate.

These results of our investigations furnish a striking proof of the fact that compounds or atoms in their nascent states have very different properties than they have after they have been aged. With an appreciation of this fact, many of the mysteries connected with some of the intensive chemical reactions occurring in living cells at the ordinary body temperatures disappear entirely. We shall not discuss here the mechanisms of the oxidation and reduction, as they occur in the natural processes, with any great detail. It is most probable that, at the moment of formation, the ferrous hydroxide-peroxide acts so as to dissociate water, and forms a strongly activated oxygen and atomic hydrogen. This process of activation may be represented by

$$\left[\begin{matrix}O_2\\ Fe\ (OH_2)_4\end{matrix}\right](OH)_2 \rightarrow \left[\begin{matrix}O_2.HOH\\ Fe\ (OH_2)_5\end{matrix}\right](OH)_2 \rightarrow$$

$$\left[\begin{matrix}OH\\ Fe\ (OH_2)_5\end{matrix}\right](OH)_2 + O_2 + H$$

If we assume this we have a natural explanation of the double rôle played by the freshly precipitated ferrous hydroxide-peroxide in its strong oxidizing action and its strong reducing action.

An analogous chemical action takes place when molecular oxygen comes in contact with the *aged* ferrous hydroxide or ferrous bicarbonate. But there is a difference in the degree of the activation. In this case the activated oxygen which is formed does not have such a high oxidation potential and can only oxidize those compounds having hydrogen which is labile and which is easily removed.

Similar relations can be followed for the stages of activation of hydrogen. There are many examples to show that when one dissociates water one obtains hydrogen of a very wide range of reducing power, according to the chemical or physical condition of the metal used for the dissociation. The chemical activity of hydrogen obtained by the dissociation of water varies enormously and the process is very complicated.

If aged ferrous hydroxide or the magnetite obtained therefrom is autoxidized in the presence of lactic acid or of potassium nitrate, both of these substances remain unchanged, although molecular oxygen is activated in some degree. There exist, between the fresh and the aged ferrous hydroxide, differences which are analogous to the differences found between the "active" and the "inactive" forms of ferric oxide, $Fe_2O_3$. We have found that active ferric oxide decomposes hydrogen peroxide, yielding an active oxygen which oxidizes benzidine. The inactive ferric oxide, on the other hand, on decomposing hydrogen peroxide, yields only the molecular oxygen which does not oxidize the benzidine. Ferrous hydroxide freshly precipitated in the presence of oxygen can oxidize lactic acid by the removal of its hydrogen. Aged ferrous hydroxide, on the other hand, can remove only labile hydrogen atoms, such as are found in paraphenylenediamine, for example.

It is our intention to study certain of the physical properties of those iron compounds which activate oxygen, and to attempt to correlate the activation to their molecular and atomic structure. At the present time we really know very little about the adsorption and the activation of gases. This statement is particularly true of those with which we are concerned here, oxygen

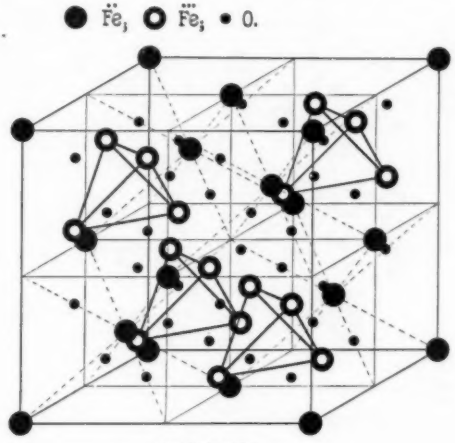


XUM

and hydrogen. It is to be expected that with more detailed information as to the structure of these iron compounds we can come to an understanding as to their adsorbing action towards various gases. The activation of the gases is, however, most probably a complex change taking place in the atoms themselves and just beyond the reach of the present experimental methods. Just now the prospects are very poor for getting the information which will enable us to understand the several degrees of activation and the selective behavior of the activated oxygen or hydrogen towards other compounds. From our experiments and investigations we believe we can safely say that there are *several different kinds* of activated oxygen, and that the hitherto recognized active forms of oxygen, such as atomic oxygen or ozone, represent only the more stable forms.

Now, as to the nature of the changes taking place in the ferrous hydroxide or ferrous bicarbonate, or the ferrous hydroxide-peroxide, during the time of aging we have little or no information which has been obtained from these substances themselves. But they may be expected to be of the same nature as those which have been found to take place in aluminum hydroxide; namely, a gradual passing, during the aging, from an apparently amorphous to a very definite crystalline form. During the aging process there occurs an ordered arrangement of the forces among the iron atoms and the hydroxyl groups, and small crystals are formed which retain some of the properties that the substances had in the amorphous state, but have lost others. The ferro-ferric oxide, $Fe_3O_4$, which has so often been mentioned, is a known and striking example of such a case. If the attempt is made to use the newly prepared oxide, $Fe_3O_4$, for the purpose of reducing potassium nitrate, the result is always absolutely negative. *But it can absorb more oxygen*, although it already has a perfectly definite crystal structure (6), such as is shown in Fig. 4; and the structure is not disarranged during this increase of the crystal's oxygen content. On examination of the interatomic dimensions involved, it was found that room can actually be found in the structure, shown in Fig. 4, for sufficient oxygen to convert the magnetite, $Fe_3O_4$, into the ferric oxide $Fe_2O_3$, without changing the arrangement of the iron and oxygen atoms already in place.

It is noticed that the unit cube (Fig. 4) contains 8 molecules, so that $Fe_3O_4$ may be written $Fe_{24}O_{32}$. After oxidation to $Fe_2O_3$ the corresponding composition would be $Fe_{24}O_{36}$. We must find room for 4 additional oxygen atoms in the cube. The first places to suggest themselves are the centers of the tetrahedra formed by the trivalent iron atoms, and they are thus shown in Fig. 5. In view of the uncertainties as to the actual radii of combination, it is quite possible that the extra oxygen atoms are placed, as shown, without undue distortion of the system. The




MAGNETITE

FIG. 4.

edge of the unit cube has a length $a = 8.30$ Å. u. and the distance from the center of the tetrahedron formed by trivalent iron atoms to each corner is

$$\frac{a}{8}\sqrt{3} = 1.795 \text{ Å.u.}$$

The radius of the oxygen atom is usually taken to be 0.65 Å. u. This leaves 1.145 Å. u. as a remainder for the radius of the iron atom. The radius of the iron atom appears to be variable and values as low as 1.25 Å. u. have been found in the case of pyrites (7) and in iron itself.

An alternative and equivalent arrangement would be to place an oxygen atom at the middle of each edge of the cube and one in the center. The room at our disposal would be the same. The question is not considered here. We are here concerned only with the possibility of putting more oxygen in $Fe_3O_4$ so as to convert it to $Fe_2O_3$ without changing the existing arrangement of the atoms. And we are especially concerned with the fact that we are dealing with a known substance which has the prop-

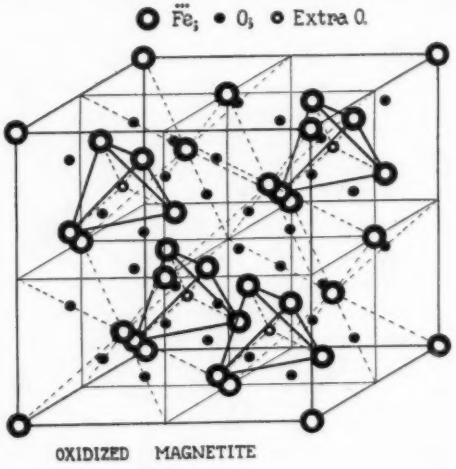



FIG. 5.

erty we have assumed for the precipitated ferrous hydroxide and ferrous carbonate. It is that, while becoming crystalline, it loses certain specific properties, such as its reducing and oxidizing powers, and yet remains capable of absorbing still more oxygen.

It must be explained here that the structure of $Fe_2O_3$ in Fig. 5 is not the most stable form. $Fe_2O_3$ of that structure is obtained by oxidizing $Fe_3O_4$ in oxygen at a temperature of about 200°C. On reaching a temperature of 550°C. or more the structure becomes unstable and goes over into the more stable calcite-like structure usually found for $Fe_2O_3$ as shown in Fig. 6. The most recent determination of this stable structure is that of Pauling and Hendricks (8).

XUM

It is our intention to proceed further with this work on the aging of ferrous hydroxide and ferrous carbonate. In particular

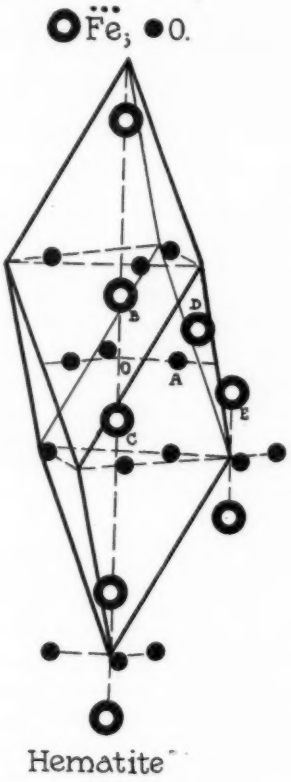



FIG. 6.

it is our plan to follow by X-ray analysis the conversion of the hydroxide or the carbonate from the apparently amorphous to the crystalline condition.

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XUM

# ON THE AGING OF NATURAL MINERAL WATERS.

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(Received for publication, May 21, 1925.)

It is a well known fact that certain mineral springs have very special therapeutic properties at the places where they appear and that these properties are often completely lost when the waters are allowed to stand for a few days or even for only a few hours before they are used. That these springs have special curative properties has been popular knowledge for centuries and in most cases the information has been transmitted from generation to generation by word of mouth. It may be said that the very wide popular belief takes on the character of a legend. As an example, we may cite the case of a certain spring whose waters cured diseases of the eye provided they were used frequently as eye-washes.

The reason, however, why the mineral waters were curative remained entirely obscure and it was particularly difficult to account for the fact that it was only the fresh water which could cure the ailments. In fact, it was doubted in medical and scientific circles that the curative properties disappeared with time. These circles were disposed to believe that the aging phenomenon merely represented a false but firmly rooted belief among people and to look upon it as a legend. With the discovery of radioactivity the doubt was resolved in favor of the general belief. It became possible, by exact measurements, to demonstrate the presence of radium emanation in certain healing waters. Since the emanation had a limited life and ultimately disappeared, it was possible to explain the fact that the waters soon lost their healing powers. This explanation proved, in many cases, to be the true one. Many mineral springs long known to be healing were found to be strongly radioactive. One of us is personally familiar with such a spring near Franzensbad in Bohemia and has



XUM

become convinced as to the truth of the claims and statements made for it.

But the question was not completely solved by referring all the curative qualities and their peculiar disappearance with time to radioactivity. There are many healing springs, especially those containing iron, which have curative properties only when the water is taken fresh, and yet show no radioactivity. It was then natural to ascribe the healing action to the presence of dissolved mineral salts. With certain reservations, this is the true explanation. It was difficult, however, to reconcile this explanation with the fact that when the mineral salts are first isolated and again redissolved, the new mineral water shows very little and, in many cases, absolutely no healing action. Then again there was the difficulty that it was often impossible to bring the isolated mineral contents back into a solution. It was found that even when the minerals were crystallized out of solution by the boiling off of the water with a high vacuum, with consequent low temperatures, they suffered a change of some sort so that they could not be completely redissolved. In particular, it was the iron, the calcium, and the magnesium which could not be entirely redissolved from the solid state. This was true even if the free carbon dioxide originally present in the mineral water was again introduced into the water used in redissolving the salts.

All of these questions have often been studied and considered because the question of transportation of mineral waters and their more extended use is closely connected with this problem of the disappearance of the healing qualities. It is now a clearly established fact that in the non-radioactive springs, the therapeutic property is very much greater at the places where they appear and if the water is used immediately after it appears above ground. It is clear, too, that the mineral contents alone are not the sole characteristics which determine the healing properties, but that a hitherto unrecognized factor plays a determining rôle.

We have mentioned and emphasized the fact that the mineral salts isolated from a healing water cannot be entirely redissolved. From this it is natural to assume that chemical changes take place in these minerals during the time that the water in which they are dissolved is being driven off. In waters which contain iron one always observes clearly and strikingly the fact that

ferric hydroxide is precipitated when the water is exposed to air for a short time. The precipitation of the calcium and eventually of the magnesium follows when the iron has disappeared from the solution.

It is really easy to understand how a mineral water loses its specific curative properties with time when it can be shown that the aging of the water can be correlated with the entire precipitation and elimination from solution of solid substances such as $Fe(OH)_3$ and $CaCO_3$. From many observations and from very old balneotherapeutic data it is known, however, that fresh spring waters often lose their curative powers even when no, or very slight, visible changes take place.

R. Glénard found, during his investigation of the mineral water from Vichy, that the fresh water had certain catalytic properties. It was, for example, capable of dissociating hydrogen peroxide into water and oxygen. But this catalytic property of the Vichy water disappeared very rapidly with time. Glénard remained content with the knowledge of this fact as the important difference between the fresh water just out of the earth and that which had been transported and aged. K. Zörkendörfer found the same to be true of the water from the mineral springs of Franzensbad. Nevertheless, the fundamental reason why the fresh waters, only, possessed these catalytic properties remained unknown.

During a sojourn at Franzensbad, one of us took up this question, since our observations on the rapid aging of solid and dissolved ferrous bicarbonate furnished us with an experimental basis for a possible clearing up of this important phenomenon. Our experimental work had made it clear *that aged ferrous bicarbonate had lost certain specific properties which it possessed while still fresh whereas still other properties were retained.* In the work with the natural mineral waters it was of particular moment to see if the aging took place in the absence of oxygen as well as in its presence.

The so called Glauberquelle III was studied and the water was found to have the chemical constituents shown in Table I.

All of the iron in this water is present, as Zörkendörfer found, in the form of ferrous bicarbonate. In connection with our work on the aging of ferrous bicarbonate in the absence of air it was of

importance to see whether or not the mineral water showed this aging when air was excluded. The experiments which were tried and which will now be described proved that the aging occurred when air was excluded. It was found, in addition, *that light accelerated the aging very greatly.*

The Glauberquellen in Franzensbad appear as fountains and the pressure of carbon dioxide in the water beneath the surface of the earth is sufficient to cause the fountains to be several meters high. The water is saturated with carbon dioxide and is crystal-clear. If the water is left in an open vessel, the larger part of the $CO_2$ gas comes off in the short time of 20 or 30 minutes and the water itself begins to opalesce. This opalescence is

TABLE I.

| In 1 kg. of water. | Glauberquelle III. |
|---|---|
| | gm. |
| Potassium ion | 0.1065 |
| Sodium " | 3.7420 |
| Calcium " | 0.2284 |
| Magnesium " | 0.0460 |
| Ferrous " | 0.0179 |
| Chlorine " | 1.5180 |
| Sulfate " | 5.0050 |
| $HCO_3$ " | 2.0880 |
| $H_2SiO_3$ " | 0.1850 |
| Free $CO_2$ " | 1.6090 |

always the first indication of a precipitation of ferric salts. The chemical changes taking place during this hydrolysis and autoxidation were heretofore always considered to be

$$Fe(HCO_3)_2 \rightarrow Fe(OH)_2 + CO_2$$

$$2Fe(OH)_2 + 2H_2O \rightarrow 2Fe(OH)_3 + H_2O_2$$

In order to see if the aging also took place in the absence of oxygen, the fresh spring water was drawn into evacuated ampules which were promptly sealed after being filled. Some of the ampules were left exposed to sunlight and the others were kept in the dark, care being taken that those exposed to sunlight were cooled off in order to eliminate possible temperature effects.

The ampules exposed to sunlight showed opalescence within 20

or 25 minutes and after an hour or two yellowish white flakes began to settle out of the water. The water in the ampules was under considerable $CO_2$ pressure since the fresh water was supersaturated with this gas. The corresponding ampules which had been kept in the dark were still crystal-clear and it was only after a period of 24 hours that the yellowish white flaky precipitate began to appear.

The experiments were often repeated and with consistent results. It was thus demonstrated that the action of light hastens the process of aging. Further, it is to be seen from the experiments that even in the presence of carbon dioxide and in the absence of air, chemical changes take place, causing the ferrous carbonate to be precipitated out and the water to lose its catalytic properties. The salts dissolved in the water as they come out of the deep interior of the ground are in a *particularly labile configuration*, a condition which it has become possible to understand only lately, because of modern knowledge regarding complex compounds and crystal structures. In this aging process, in the dissolved as well as in the solid salts, we have to do with the rearrangement in space of the atoms, of the molecules, and of groups of molecules. It is fortunate that the rearrangement takes place so slowly that it can be recognized as a process of aging.

In this work we were concerned with the more detailed study of this spatial rearrangement or aging; for with a better understanding of the process we could also hope to gain an insight into the therapeutic action of iron-containing mineral springs. An experimental study of this question faces the difficulty that in mineral waters we have only very small quantities of iron. The fact that the quantity of iron is small is, however, no reason to doubt that the iron has a very definite therapeutic action when in the "active" form. On the other hand, if the water has had time to age the iron becomes "inactive" and the therapeutic quality disappears.

As "active" iron we designate those iron ions or iron molecules which give the following characteristic reactions.

(1). The blood test. A blue color appears in a mixture of benzidine hydrochloride and hydrogen peroxide. (Peroxidase action.)
(2). The property of absorbing molecular oxygen. (Oxidase action.)
(3). Intense decomposition of hydrogen peroxide. (Catalase action.)

(4). The property of aiding bacterial growth; that of *Bacterium lepisepticum* being an example. (Biological catalysis.)

None of these reactions are given by "inactive" iron ions or iron molecules. This seems to be a suitable place to emphasize the fact that the question of whether or not the iron is "active" does not depend on its valence. We have found that the divalent iron, $\ddot{Fe}$, and the trivalent iron, $\dddot{Fe}$, can each appear in both the active and inactive forms. This fact appears most clearly and simply in the case of the active and inactive solid ferric oxide, $Fe_2O_3$ (first described by Welo and Baudisch). *The active $Fe_2O_3$ shows all of the characteristic reactions in the foregoing list and the inactive form shows none of them although the two forms of the oxide are of identical chemical composition.* In the special case of the mineral springs, we are dealing, however, with the dissolved compounds and not with salts in the solid state. We have found that analogous relations as to activity and inactivity hold for solutions, in water, of compounds of the following composition.

$$\left[\ddot{Fe}\begin{matrix}OH_2\\(NC)_5\end{matrix}\right]Na_3 \qquad \text{and} \qquad \left[\dddot{Fe}\begin{matrix}OH_2\\(NC)_5\end{matrix}\right]Na_2$$

Sodium pentacyano-aquo-ferroate. Sodium pentacyano-aquo-ferriate.

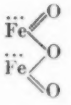
Even in dilutions of 1 part in 500,000 both of these salts give intensive benzidine reactions, they absorb oxygen and further the growth of *Bacterium lepisepticum* as Webster (1924–25) has shown. Both of these relatively simple complex compounds of iron behave in many respects like hemoglobin.

In order to imitate the conditions existing in mineral waters as closely as possible we were concerned mainly with the study of the aging of ferrous bicarbonate. The results are given in considerable detail in the preceding paper. In that paper it is shown that freshly precipitated ferrous bicarbonate undergoes an extremely rapid aging process. For example, the green precipitate formed when sodium bicarbonate is added to ferrous sulfate in the presence of air gives a marked benzidine reaction. This ability to give the benzidine reaction disappears, however, in the course of a few minutes. In this connection Just and Günzburg have also made an important observation. They found that ferrous bicarbonate synthesized from metallic iron and carbon dioxide

XUM

has a higher solubility in water when first formed. After an aging of a few minutes it is partly separated out of its solution.

Our old valence formulas do not permit us to predict the degree of solubility of a compound or its general chemical behavior. The formula



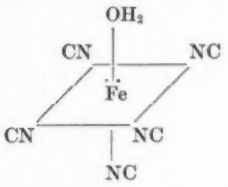
for example, expresses only the analytical relations which are found to exist between the iron and the oxygen. In order to be nearer a concept of the actual physical facts we must think of the compound $Fe_2O_3$ as a space lattice; as a stoichiometric and ordered arrangement among themselves of 2 $n$ atoms of iron and 3 $n$ atoms of oxygen. In our other paper we have considered this matter, especially as applied to the active and inactive forms of the oxide $Fe_2O_3$, and have demonstrated that the chemical and biological action is, in some way, related to the arrangement of the atoms in the lattice.

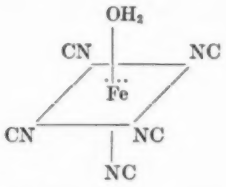
Here we are interested in seeing whether or not like considerations as to the relation between chemical properties and crystal structure can be carried over and applied to *dissolved* iron salts. We find that the question can be answered in the affirmative and in the succeeding discussion we shall consider the evidence.

It is known from the work of Scherrer and Stoll (1922) and others on the crystal structures of potassium chloroplatinate, $[PtCl_6]K_2$, and nickel chloride hexammonate, $[Ni(NH_3)_6]Cl_2$, that the Werner coordination formulas represent the true groupings of the atoms in these complex compounds. In the former compound the platinum ion is surrounded octahedrally by 6 chlorine ions and this group, $PtCl_6$, acts as a coordination center for 8 potassium ions arranged at the corners of a cube. Similarly, in nickel chloride hexammonate we have correspondence with the Werner coordination scheme since the 6 $NH_4$ molecules or groups form an octahedron about the nickel; the group $Ni(NH_3)_6$ being, in its turn, a coordination center for the chlorine ions placed at the corners of a cube.

Entirely analogous relations exist in the pentacyano compounds

of iron which we have mentioned. The arrangement of the complex ions can be represented as follows, according to the Werner scheme.




These pentacyano complexes differ from the corresponding ferro- and ferricyanide complexes only in that 1 molecule of water has been substituted for one of the cyanide groups.

While the ferrocyanides and ferricyanides prove to be inactive iron salts when tested by the four reactions we have enumerated, the corresponding pentacyano salts are extremely active and are similar, in many respects, to hemoglobin. However, an inactive ferrocyanide is rapidly changed by the influence of sunlight into the active pentacyano compound, as Baudisch and Bass (1922) have shown. On continued illumination the photochemical change proceeds too far and the pentacyano compound is changed into sodium cyanide and inactive iron ions.

This action of light on the inner sphere of certain complex iron salts is readily correlated to the results of our experiments with mineral waters exposed to light.

In the *active* ferrous bicarbonate dissolved in mineral water the specific catalytic action may be assumed to be, in some way, connected with the spatial arrangement of the $HCO_3$ and the $OH_2$ groups around the central iron atom in a manner such as may be represented by the formula

$$\begin{bmatrix} O_3CH & .. & OH_2 \\ O_3CH & Fe & HCO_3 \\ O_3CH & & HCO_3 \end{bmatrix} Na_3$$

Sodium pentabicarbonate-aquo-ferroate.

which is analogous to

$$\begin{bmatrix} CN & .. & OH_2 \\ CN & Fe & NC \\ CN & & NC \end{bmatrix} Na_4$$

Sodium pentacyano-aquo-ferroate.

With the rearrangement of the molecules in the inner sphere, brought about either by heat, light, or simply by aging, the central iron atom of these complex salts loses its special "active"

XUM

property and finally ferrous carbonate or hydroxide is precipitated. However, if oxygen enters into the reaction, the complex ferrous salts are quickly decomposed and the iron quantitatively precipitated as red ferric hydroxide.

On the bases of these simple analogies we have thus come to a plausible explanation as to why mineral waters possess certain catalytic properties which disappear in the course of a short time. At the same time there is obtained an insight into the mechanism of the therapeutic action and an understanding as to the way in which the curative properties vanish when the water is allowed to stand. We can assume that in the same way or in a similar way that an active iron salt such as hemoglobin aids the growth of certain bacteria, the active iron salts in fresh mineral waters aid the life processes of body cells as soon as the salts enter the stomach and intestines and begin to take part in the metabolism. We do not believe that this life-aiding action is confined to the iron, alone, of all the minerals in these healing springs. It is rather to be expected that all of the salts dissolved in the water play a part. After iron, it is likely that calcium will be found to be the most important.

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XUM

XUM

# THE PHOSPHATIDE AND TOTAL PHOSPHORUS CONTENT OF WOMAN'S AND COW'S MILK.

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(Received for publication, May 11, 1925.)

Recent studies have made it evident that phosphorus plays a more important rôle in the metabolism of infants, more especially in the metabolism of bone, than had been realized. It is therefore of increasing interest to inquire closely as to the distribution of the phosphorus in the dietary of the growing child. This naturally implies a study of milk, which forms the sole constituent of the dietary throughout the first 6 months of life and its basis during the following year. Investigations of the total phosphorus of milk have been numerous, whereas studies of its phosphatide fraction have been very limited, and, for the most part, were carried out many years ago. The significance of phosphatide in the diet is still a mooted question; it should be borne in mind, however, that some investigators regard it as an essential and irreplaceable dietetic factor. Whatever may be the view-point in this regard, it must be remembered that it is a constituent of almost all the cells of the body, and that in the human blood organic phosphorus constitutes over 80 per cent of the total phosphorus, and inorganic phosphorus only 3 to 4 mg. per cent. Studies of the phosphatide of milk have given most varied results, which is not surprising considering the diversity of the methods employed. It would serve no purpose to enumerate or review these investigations in detail. The most reliable is probably that of Koch and Woods published in 1905 (1). The superiority of this work rests on the fact that the milk was not dried and that care was exercised not to include any water-soluble extractives, inorganic phosphates, phosphorus in simple organic combination, and inorganic salts in the final precipitation. Since these analyses



XUM

were carried out, however, newer methods of determining phosphorus have been devised, especially in connection with the examination of blood. It is these methods which seemed, when used in combination, to be well suited for the investigation of milk.

For extracting the phosphatide we made use of Bloor's (2) method, which is especially adapted for the analysis of fluids, and possesses the advantage over the old Soxhlet method—employed by most investigators—of not necessitating drying the preparation. For estimating the phosphorus content of the phosphatide which had been extracted the colorimetric method of Tisdall (3) was employed. This was found to have certain advantages for our purposes over the methods of Bell and Doisy and of Briggs, which are so useful in examining blood plasma. In the first place, the Tisdall method does not necessitate the immediate completion of the test, and the procedure may be interrupted at any of several stages without any danger of higher colorimeter readings. Moreover, the fact that the amount of solution available for comparison with the standard is greater than 15 cc., the maximum used in the Briggs' test, constitutes a decided advantage in testing milk. It will be noted that it was found necessary to modify the standard methods in a few particulars in order to obtain results that checked on duplicate solution. The procedure for determining the phosphatide in milk was as follows:

The method, broadly stated, consists in the extraction of the phosphatides from the milk with a warm alcohol-ether solution as suggested by Bloor, and the determination of the amount of phosphorus present after digestion by the Tisdall method for inorganic phosphorus. The samples of human milk were obtained by means of the breast-pump from about the mid-period in the nursing.

*a. Extraction of Phosphatides.*—5 cc. of well mixed milk is run slowly with *continuous shaking* into a 100 cc. volumetric flask containing 30 to 40 cc. of a mixture of 3 parts of alcohol and 1 part of ether (both redistilled). The flask is immersed in hot water for 2 minutes with constant shaking, and then cooled immediately under cold water to room temperature. The alcohol-ether solution is made up to volume, mixed, and filtered.

*b. Digestion.*—10 cc. of the alcohol-ether extract are evaporated to complete dryness in a small digestion flask in a water bath. 5 drops (3

drops then 2) of concentrated $H_2SO_4$ are added; this is boiled 2 or 3 minutes, a few drops of concentrated $HNO_3$ are added; this is boiled until colorless. The ashing should require about 10 minutes. Then 5 cc. of distilled water are added and boiled 3 minutes. It is washed into a 15 cc. test-tube, using 5 cc. of water.

*c. Neutralization and Precipitation.*—While the solution is still warm add 1 drop of a 1 per cent alizarin solution (indicator). Make just alkaline with concentrated $NH_4OH$, and bring back to neutrality by adding a weak $HNO_3$ solution, drop by drop. Add 2 cc. of the strychnine solution and let stand until precipitation is complete (usually ½ hour is sufficient).[1]

*d. Color Development.*—Centrifuge and pour off supernatant liquid, washing three times with distilled water and centrifuging after each washing. Dissolve precipitate in 2 cc. of a 1 per cent NaOH solution. Add water to 10 cc. and transfer to a 100 cc. volumetric flask, washing it three times with 10 cc. of $H_2O$. Add 20 cc. of a 20 per cent potassium ferrocyanide solution and 10 cc. of concentrated HCl (accurately measured). Mix, let stand 10 minutes, dilute to mark, and read in the colorimeter.

*Preparation of Standard.*—For this purpose the standard used by Briggs (4) for blood was employed; 2.5 cc. of a solution of $KH_2PO_4$ containing 1 mg. of P per 100 cc. is pipetted into a test-tube and precipitated at the same time as the unknown solution, keeping the dilution the same in both.

*Calculation.*—

$$\frac{20}{R} \times \frac{\text{Dilution of unknown}}{\text{Dilution of standard}} \times \frac{100}{\text{Actual amount of milk used}} \times 0.25 = \text{Mg. P per 100 cc. milk}$$

## DISCUSSION.

Considerable difficulty was encountered in obtaining results which checked. Duplicate extractions of the phosphatides were made, as well as duplicate determinations on each extraction. After working with a solution containing a known amount of phosphorus and still getting irregular results, several changes

[1] Tisdall's solutions for the determination of inorganic phosphorus were used throughout.

The strychnine nitrate-precipitating reagent was modified as follows:

*Solution A.*—50 gm. of ammonium molybdate in 150 cc. of $H_2O$, using 100 cc. as 1 volume.

*Solution B.*—2 volumes of concentrated $HNO_3$ and 1 volume of $H_2O$, using 300 cc. as 3 volumes.

*Solution C.*—1 volume of Solution A and 3 volumes of Solution B, using 300 cc. as 3 volumes.

*Solution D.*—2 gm. of strychnine nitrate in 100 cc. of $H_2O$; 1 volume of Solution D poured into 3 volumes of Solution C—total 400 cc.

XUM

were introduced which gave concordant results. To 5 cc. of the standard $KH_2PO_4$ solution (1 mg. of P per 100 cc.) 5 drops of concentrated $H_2SO_4$ were added in one tube, and 3 drops of $H_2SO_4$ in another; both were neutralized with $NH_4OH$ and weak $HNO_3$, and precipitated with Tisdall's reagent. The color was developed and compared in the colorimeter with a 0.05 mg. P standard set at 20, containing no $H_2SO_4$ or neutralizing solutions. The tube containing 5 drops of $H_2SO_4$ read 19, whereas the tube containing 3 drops of $H_2SO_4$ read 19.9 compared with the standard of 20. Neutralizing with NaOH in place of $NH_4OH$ was tried with the same result. Neutralizing with $NH_4OH$ and adding different quantities of saturated $NH_4NO_3$ (3 and 5 cc.) gave similar results, the tube containing the 5 drops of $H_2SO_4$ always giving too low a reading, 18 to 18.5 compared to the standard set at 20. The tube containing 3 drops of $H_2SO_4$ read as it should, 20 as against 20. Finally 0.5 cc. of a strychnine nitrate solution (1 gm. of strychnine nitrate in 100 cc. of $H_2O$ and 4 cc. of $HNO_3$) was added to a tube containing 5 drops of $H_2SO_4$ in addition to the neutralizing, precipitating, and color-developing reagents. This mixture was then compared in the colorimeter with the standard. The solutions matched exactly, both reading 20. 1 cc. of the additional strychnine nitrate solution gave the same result as 0.5 cc., the reading again being 20. $H_2SO_4$ alone with no neutralization by $NH_4OH$ and $HNO_3$ gave readings varying from 19.6 to 21.1; whereas $H_2SO_4$ with neutralization read 22.1.

The precipitating reagent of Tisdall was then modified so as to contain 0.04 gm. of strychnine nitrate per 2 cc. instead of 0.03 gm. per 2 cc.; the additional strychnine nitrate being necessary to compensate for the 5 drops of $H_2SO_4$ used in ashing. We wish to thank Dr. T. F. Zucker for this helpful suggestion.

Bloor's method for extraction of phosphatides from serum or plasma with redistilled alcohol and ether was followed instead of using anhydrous solvents as we did not find, contrary to the experience of Collison (5), the amount of inorganic phosphorus present in the solvent after extraction to be of estimative quantity. We carried out Koch and Woods' (1) advice of neutralizing with $NH_4OH$ and $HNO_3$ after ashing and boiling before precipitation. Meigs, Blatherwick, and Cary (6) precipitate P *with*

XUM

TABLE I.

*Lipoid Phosphorus in Milk.**

| Author. | Date. | Woman's milk. | | Cow's milk. | | Stage of lactation. | Method used. | Remarks. |
|---|---|---|---|---|---|---|---|---|
| | | No. of analyses. | Amount. | No. of analyses. | Amount. | | | |
| | | | *mg. per 100 cc.* | | *mg. per 100 cc.* | | | |
| Schlossmann. | 1897 | Not stated. | 6.4 | Not stated. | 3.9 | Not stated. | Soxhlet extraction. | |
| Stoklasa. | 1897 | " " | 6.80 | " " | 3.6 | " " | " " | Extremes. |
| | | | 7.44 | | 4.52 | | | |
| Burow. | 1900 | 9 | 2.33 | 4 | 2.16 | " " | Equal parts alcohol and ether. | |
| Ellenberger. | 1902 | Not stated. | 2.4 | Not stated. | 2.0 | " " | Not stated. | |
| Koch and Woods. | 1905 | 1 | 3.12 | 3 | 3.08 | " " | Chloroform precipitate. | |
| Nerking and Haensel. | 1908 | 10 | 0.96 | 17 | 1.45 | " " | Soxhlet extraction. | Extremes. |
| | | | 3.19 | | 4.65 | | | |
| Glikin. | 1909 | 1 | 5.31 | 2 | 2.06 | " " | " " | |
| | | | | 1 | 4.69 | | | |

* These data represent 4 per cent of the figures reported for lecithin.

TABLE II.

*Phosphatide and Total Phosphorus in Woman's Milk ("Early Period").*

| Case No. | Date. | Race. | Age of infant. | Lipoid P. | Total P. | Fat. | Interval previous to examination. | Remarks. |
|---|---|---|---|---|---|---|---|---|
| | *1924* | | *days* | *mg. per 100 cc.* | *mg. per 100 cc.* | *per cent* | | |
| 14 | June 24 | Negro. | 2 | 2.54 | 8.16 | 2 | None. | Deep yellow colostrum. |
| 4 | Mar. 6 | White. | 6 | 1.48 | 8.77 | | " | Expressed manually. |
| 5 | " 11 | " | 7 | 2.59 | 16.90 | | " | |
| 2 | Feb. 27 | Negro. | 7 | 2.95 | | | " | |
| 4 | Mar. 8 | White. | 8 | 1.72 | | | " | Same case as Mar. 6, 1924. |
| 6 | Apr. 14 | Negro. | 10 | 4.06 | 17.02 | 2 | " | |
| 2 | Mar. 3 | " | 12 | 3.55 | | | " | " " " Feb. 27, 1924. |
| 3 | " 1 | White. | 14 | 3.30 | | | " | |
| 7 | Apr. 23 | " | 14 | 2.81 | 14.41 | 4 | " | |
| | | | | 2.77 | | | 48 hrs. | |

TABLE III.

*Phosphatide and Total Phosphorus in Woman's Milk ("Mature Period").*

| Case No. | Date. | Race. | Age of infant. | Lipoid P. | Total P. | Fat. | Interval previous to examination. | Remarks. |
|---|---|---|---|---|---|---|---|---|
| | *1924* | | *mos.* | *mg. per 100 cc.* | *mg. per 100 cc.* | *per cent* | | |
| 1 | Mar. 5 | White. | 8 | 2.10 | | | None. | Expressed manually. |
| 8 | Apr. 29 | " | 3½ | 2.96 | 19.27 | 4 | " | |
| 9 | May 7 | Negro. | 3 | 2.67 | 13.95 | 3 | " | |
| 10 | " 14 | White. | 2-9 | 2.67 | 24.39 | 4 | 12 hrs. | Composite sample from 10 women. |
| 11 | " 20 | " | 1½-10 | 2.25 | 16.0 | 4 | 18 " | " " " 10 " |
| 12 | " 28 | " | 1½-10 | 2.38 | 13.18 | 4 | 12 " | " " " 10 " |
| 13 | June 3 | " | 1½-10 | 2.28 | 12.34 | 5 | 12 " | " " " 10 " 576 mg. total nitrogen per 100 cc. milk. |

a hot solution, whereas it will be noted that we precipitated *in* a warm solution; this procedure was adopted as it was found to bring down the precipitate more quickly.

The results obtained by previous investigators in regard to the phosphatide content of woman's milk, as well as those obtained by us, are summarized in the accompanying tables (Tables I, II, and III). In general the figures are in agreement. On reviewng our data it became evident that the cases should be divided into two groups, those of the "early period," comprising mothers whose infants were under 2 weeks of age, and those of the "mature period," where the infants were from 6 weeks to 10 months of age. Between these two groups there was a marked distinction. Those tested during the early period gave irregular and inconstant results as compared to the later group, the percentages varying from 1.48 to 4.08 mg. This irregularity is probably partly to be accounted for by a variable admixture of colostrum, as well as by the fact that the function of the breasts has not yet become stabilized. The phosphatide from milk obtained during the mature period was, on the other hand, fairly constant, ranging from 2.09 to 2.96 mg. For the most part the samples represented milk from individual women, in some instances, however (designated in Table III as "composite samples") they were portions of the combined 24 hour milk secretion of ten women.[2] No reciprocal relationship could be established between the amount of phosphatide in woman's milk and its total content of phosphorus or its percentage of fat. For example, Table III shows that the milk of Case 10 contained almost 100 per cent more total phosphorus than that of Case 13, whereas the phosphatide content was, respectively, 2.67 and 2.28 mg. Likewise although the percentage of fat in specimen No. 9 was 3 per cent and that of No. 13 was 5 per cent, the phosphatide content of the former was 2.67 mg. and of the latter 2.28 mg. Furthermore, no distinction in phosphatide content was noted between the milk of white and of negro women. One specimen

[2] These composite samples were obtained from the milk station of the Children's Welfare Federation which furnishes breast milk for babies. The individual specimens were from the adjoining Sloane Hospital for Women. We wish to take this opportunity to thank them for their helpful cooperation.

XUM

of colostrum was examined. This term should designate milk which is decidedly yellow and contains a large number of colostrum corpuscles, not merely the milk of the 1st week or 10 days of lactation. The phosphatide of this specimen fell within normal limits, although its total phosphorus content was exceptionally low (8.16 mg.).

A considerable number of samples of cow's milk were analyzed for their content of phosphatide (Table IV). Some of these were raw and others had been pasteurized, a factor which did not affect the results. The percentage in cow's milk varied from 5.29 to 6.99 mg.; the amount was about twice that obtained in woman's milk. Two specimens of cream and two of centrifugalized skimmed milk were tested (Table IV). The skimmed milk, which contained only 0.4 per cent of fat, gave the lowest figures which we obtained for phosphatide (4.12 and 4.44 mg.), and the cream containing 32 and 20 per cent of fat was found to have 10.25 and 7.14 mg. respectively of phosphatide. In view of these results, which represent the lowest and uppermost extremes in regard to the fat content of milk, we must infer that there is some association or relationship between fat and phosphatide. Possibly this was exaggerated in these samples by the centrifugalization. The total phosphorus of the milk ran in inverse ratio to the phosphatide content; in other words, it was highest in the skimmed milk and lowest in the two samples of cream.

The opportunity presented itself of examining the milk of two goats and an ass. The results of these analyses are given in Table V, and are too few to warrant drawing any conclusions. The total phosphorus content of goat's milk was found exceptionally high, as has been reported by others. The same is, however, not true of its phosphatide content. In regard to the tests of the ass' milk the only point worthy of mention is the low phosphatide in one test (2.38 mg.) associated with a low fat percentage (1 per cent). Two subsequent samples of milk were obtained from this animal in order to verify the low figure for fat.

It will be observed on referring to Table I, that the range of phosphatide in cow's milk as reported by others is from 2.12 to 4.10 mg., amounts considerably lower than those obtained by us.

XUM

TABLE IV.

*Phosphatide and Total Phosphorus in Cow's Milk.*

| Nature of milk. | Dealer. | Date. | Lipoid P. | Total P. | Fat. | Approximate interval previous to examination. | Remarks. |
|---|---|---|---|---|---|---|---|
| | | *1924* | *mg. per 100 cc.* | *mg. per 100 cc.* | *per cent* | | |
| Pasteurized. | B. | Mar. 1 | 6.5 | | | 24 hrs. | |
| " | S. | Apr. 14 | 5.33 | 75.94 | 3.6 | 24 " | |
| Raw. | B. | Feb. 27 | 6.51 | | | 24 " | |
| " | W. G. | " 21 | 6.99 | | | 24 " | |
| " | " | Mar. 13 | 5.74 | 80.0 | | 24 " | Milk was diluted with an equal quantity of $H_2O$ before extracting lipoid. |
| " | " | " 13 | 5.98 | | | 24 " | |
| " | " | " 24 | 5.29 | 80.0 | 4.0 | 24 " | |
| " | " | Apr. 22 | 6.15 | 81.63 | 4.0 | 24 " | |
| " | " | " 22 | 6.06 | | | 48 " | Same milk as used in previous test. |
| Skimmed. | " | June 5 | 4.12 | 96.00 | 0.4 | 24 " | |
| " | " | " 10 | 4.44 | 109.0 | 0.4 | 24 " | |
| Cream. | " | May 22 | 10.25 | 64.93 | 32 | 24 " | |
| " | " | June 3 | 7.14 | 65.78 | 20 | 24 " | |
| Colostrum. | | " 17 | 11.76 | 93.75 | 4.2 | 18 " | Calf 2 days old. |
| " | | " 21 | 10.52 | 113.63 | 4.0 | 18 " | " 5 " " Same calf as in previous test. Sp.gr. 1.032. Total nitrogen 644 mg. per 100 cc. |

TABLE V.

*Phosphatide and Total Phosphorus of Goats' and Ass' Milk.*

| Kind of milk. | Date. | Lipoid P. | Total P. | Fat. | Length of lactation period. | Interval previous to examination. | Results. |
|---|---|---|---|---|---|---|---|
| | *1924* | *mg. per 100 cc.* | *mg. per 100 cc.* | *per cent* | | | |
| Goat I. | May 1 | 5.35 | 102.56 | 6 | 3 mos. | 24 hrs. | |
| " II. | " 1 | 3.0 | 133.33 | 6 | 3 " | 24 " | |
| Ass I. | " 13 | 7.19 | 72.72 | 3 | 2 wks. | 2 wks. | |
| " I. | " 20 | 2.38 | 60.60 | 1 | 3 " | 2 " | Milk obtained several hours after feeding. |
| " I. | " 23 | | | 1 | 3½ " | 2 " | " " 2 hrs. after feeding. |
| " I. | " 28 | | | 1 | 4 " | 2 " | 55 cc. sample taken after first portion of milking had been discarded. |

The figures are consistently lower and would seem to depend upon differences in methods of analysis. As stated, most of the tests previously employed involved prolonged boiling of the milk, which may bring about decomposition of some phosphatide. Bloor's so called "gentle treatment" for the extraction of phosphatide from plasma avoids this danger. The difference in results may be due to the practice of the majority of investigators of drying the milk, whereas we maintained it in a fluid state. In this connection it is of interest to note that Greenwald (7) in his analyses of blood for lipoid phosphorus obtained exceptionally high percentages, which he attributed to the fact that he avoided drying his material, and was, therefore, able to bring about a more complete extraction. In his opinion lipoid in the dry state is surrounded by protein which renders it less accessible to solvents. An experiment which we carried out to elucidate this point seems to favor this hypothesis. A dried whole milk powder was divided into three portions. The first, consisting of the powder as taken from the can, was added to the alcohol-ether mixture and extracted in the usual way; the second portion was dried in the oven at 100° several times, for a total period of about 5 hours, being placed in a desiccator during the intervals, and was finally brought to constant weight; the third portion was simply diluted with water in amount sufficient to bring it back to its original fluid state. The total phosphorus of the first portion—the dried milk as it is marketed—was 83.15 mg. per 100 cc. and that of the dried milk, after being brought back to its fluid state, was 83.33 mg., thus showing that the amount of water added had been correctly estimated. The results obtained for phosphatide phosphorus in the three portions were: dried milk from the original container 2.47 mg. per cent, that dried to constant weight 1.77 mg. per cent, and that which had been rendered fluid 4.91 mg. per cent. In other words, an analysis of the original dried milk showed only one-half as much phosphatide as the same milk which had been rendered liquid, and the phosphatide of milk dried to constant weight was in turn only 50 per cent as great as that of the original dried milk and only 25 per cent as great as the milk which had been fluidified. This experiment convinced us that Greenwald's experience in connection with lipoid analyses of blood applies to analyses of milk

and that drying leads to an incomplete extraction of the phosphatide fraction. Heat in itself may diminish the solubility of the lecithin.

In order to control our method, phosphatide was added to milk in the form of a solution of egg lecithin and analyses were carried out to recover it quantitatively. In this particular sample of milk tested, the phosphatide was 6.99 mg. per cent, the theoretical calculation after the egg lecithin was added was 7.22 mg. per cent, and the actual analysis showed 7.30 mg. per cent. The margin of error was, therefore, small.

A more convincing control, in view of our high figures for cow's milk, was the addition of inorganic phosphorus to the milk to determine whether inorganic salts may be dissoved by the alcohol-ether mixture. Such was found not to be the case, as Table VI shows.

TABLE VI.

| 45 cc. milk + 5 cc. known $KH_2PO_4$ solution.* | P per 100 cc. milk. | |
|---|---|---|
| | Lipoid. | Total. |
| | *mg.* | *mg.* |
| Actual results | 4.67 | 75.47 |
| Theoretical results | 4.77 | 73.00 |

* 5 cc. of solution contained 0.5 mg. of inorganic phosphorus.

As a further test, we evaporated the alcohol-ether solvent from an extract and added water to the residue to take up any inorganic phosphorus that might have been dissolved. The amount of phosphorus obtained in this way was so small that it could not be estimated.

A modification of the Koch and Woods method for lipoid P was carried out on an alcohol-ether extract from milk. 50 cc. of milk were extracted with alcohol ether. Bloor suggested the alcohol-ether extract be removed in the cold (after filtering 24 hours following extraction) under reduced pressure, and the residue dried in a vacuum over $H_2SO_4$. This residue was first extracted with petroleum ether (b.p. 40–60°) four or five times, the solution filtered, and a lipoid P determination done on the petroleum ether extract; 0.90 mg. of P per 100 cc. of milk was found in this portion. The crystalline residue remaining undissolved by

XUM

the treatment with petroleum ether was then extracted with alcohol ether three times, again using Bloor's method. The alcohol ether was filtered off and a lipoid P determination done on this extract; 1.56 mg. of P per 100 cc. of milk were found to be present. The crystalline residue remaining was treated a second time, with petroleum ether and a lipoid P determination again carried out. Only a trace of P was found, too small an amount to be determined. The entire crystalline residue was then dissolved in boiling water, washed into a flask, the water evaporated, and the residue ashed in the usual way with $H_2SO_4$ and $HNO_3$. From this ashing 0.78 mg. of P was obtained; some of this may have been non-lipoidal in nature. By adding these four results together 3.24 + mg. of P per 100 cc. of milk were found to be present.

A determination on the milk was also done by simply extracting with the alcohol-ether mixture and ashing. This showed 3.44 mg. of P per 100 cc. of milk.

As the total percentage of lipoid phosphorus by the modified Koch and Woods method is 3.24 + mg. and the lipoid phosphorus by our method is 3.44 mg., the methods would seem to check within the limit of error.

These results raise the question of the true nature of the substance which we, following Bloor and others, have termed lipoid phosphorus. Evidently the phosphorus in the alcohol-ether extract of milk is not present altogether in the form of the ordinary phosphatides, for these are soluble in petroleum ether. That the material soluble in alcohol and ether but insoluble in petroleum ether is not inorganic P is indicated by the experiments which have been described above. Since the P found in the alcohol-ether extract was shown not to consist of inorganic P, the conclusion would seem to be that it is either lipoid P alone or combined with the P from some as yet undetermined lipoid-like substance. In this connection attention may be called to the experiments of Osborne and Wakeman (8) who obtained from milk an ether-soluble phosphatide, the nature of which they could not definitely determine.

XUM

## CONCLUSION.

In view of the fact that previous studies of the phosphatide of milk have given variable results and have been carried out by various methods it seemed worth while to investigate this subject further. This seemed all the more pertinent in view of the fact that newer methods for phosphatide investigation have been recently devised.

A large number of samples of both woman's and of cow's milk were analyzed. Those of woman's milk were divided into two groups: those from the "early period" of lactation embracing the first 2 weeks after childbirth and those from the "mature period" of later months. The results of these tests confirm in the main those reported by previous authors. No relation was found to exist between the fat percentage and the content of phosphatide. There was, however, a definite distinction between the amount of phosphatide in the milk of the "early period" and that in the "mature period."

A large number of samples of cow's milk were analyzed for phosphatide and total phosphorus. These analyses showed that cow's milk contains about twice as much phosphatide as human milk. The amount was higher in milk or in cream with a high percentage of fat. However, there was no parallelism between the fat and the phosphatide content. Specimens containing a considerable amount of colostrum also showed a high percentage of phosphatide. The total phosphorus of cow's milk averaged about four times as high as that of woman's milk and was still higher in the milk of goats.

Our tests of woman's milk showed a percentage of phosphatide which is considerably higher than that found by other investigators. We believe that this was due to the solvents which were employed and to the fact that the milk was tested in a fluid rather than in a dried state. Experiments would seem to indicate that the solubility of lipoid phosphorus varies markedly according to its physical state and the solvents which are used. It is possible that the substances which we have included in our analyses are lipoid-like bodies of unknown nature.

XUM

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XUM

# THE FATTY ACIDS IN THE SUBCUTANEOUS FAT OF MAN.

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In a recent communication, Sperry and Bloor (1) have concluded that fecal fat should no longer be considered to be fat that has escaped absorption but fat excreted from within the animal organism. This conclusion was based on the fact that the composition of the excreted material is, to a considerable degree at least, independent of the food ingested. Their contention is lent support by other work reported by Bloor (2) which showed that the composition of this fecal fat is much like that of the blood lipoids. Thus the ratio of solid to liquid fatty acids of fecal fat was found to be similar to that of the fat in the circulating fluid. It is true that the iodine number of the liquid acids of the blood is somewhat higher than that of the feces, but this difference is perhaps small enough to be accounted for by the fact that the rather vigorous treatment necessitated in the isolation of the lipoids from the feces might bring about changes in the fatty acids therein. Furthermore, reduction in the lower gut is not unknown. Hence, it is reasonable to expect a hydrogenation of the highly unsaturated acids during their stay in this region.

The constancy of the composition of these two sources of fat calls to mind the fact that tissue fat is quite constant in composition for any species and is, within reasonable limits, independent of the composition of ingested fats. If fecal fat is excreted unchanged from the fat depots, then a similarity may be expected to exist between the fat feces and that of the fat depots. That this is the case has been demonstrated in the investigation reported herein, for the reason that the ratio of solid to liquid fatty acids of the fat analyzed is similar to the one found by Sperry and Bloor (1) in their study of fecal fat. The presence of unsaturated acids



XUM

other than oleic, has been demonstrated; of these, linoleic predominates. A smaller amount of an acid with four double bonds was identified and considerably less of an acid with three double bonds found. Tissue fat is thus not only like fecal fat but also similar to blood fat (3).

## EXPERIMENTAL

In a lipectomy[1] performed upon an obese individual at the University Hospital, approximately 18 pounds of subcutaneous fatty tissue were removed from the abdomen. The lipoids in this tissue served as the source of material for the present study and were extracted therefrom in the following manner.

The tissue was dissected from adhering outer layers of the skin and divided into small cubes. These were transferred to a large flask and covered with 3 liters of absolute ethyl alcohol. The mixture was then refluxed for 12 hours. This treatment resulted in the solution of a considerable portion of the lipoids in the alcohol as well as in the "trying out" of fat from the tissues by the heat of the boiling solvent. These combined solutions were decanted from the partially extracted tissue at the end of the 12 hour refluxing and fresh solvent was added to the material in the flask. This process was repeated several times and at the end of the sixth extraction with alcohol, four extractions with ethyl ether were made. These extractions yielded a practically fat-free residue, as evidenced by the fact that it was possible to pulverize it to a fine powder. As an added precaution, however, this light yellow powder was transferred to a Soxhlet apparatus and extracted first for 48 hours with absolute alcohol and then for a similar period with absolute ether. The solvents in the combined extracts were removed by distillation *in vacuo* at room temperature, the last traces being eliminated by placing the oil in a desiccator in which a vacuum of 5 mm. was maintained for 72 hours.

For analysis, the recently described methods of Sperry and Bloor (1) were used. By this means the percentages of non-saponifiable matter, solid fatty acids, and liquid fatty acids were obtained. In addition to this, a separation of the constituents of the solid fraction by the process employed by Jacobson and Holmes (4) was attempted and finally a fractionation of the unsaturated acids by a procedure first described by Farnsteiner (5) and recently modified by Brown and Beal (6).

Briefly stated the combination of these methods was as follows: 5 to 10 gm. of the dry fat were saponified with boiling alcoholic potassium hydroxide, the saponified mixture was acidified with dilute hydrochloric

[1] This material was made available for study through the courtesy of Dr. Hugh Cabot, of the Department of Surgery, and Dr. A. S. Warthin, of the Department of Pathology.

XUM

acid (1:3), and the fatty acids as well as the non-saponifiable matter were removed by extraction with petroleum ether (b.p. 30–50°C.). These were separated from each other as described by Sperry and Bloor (1) and the fatty acids dissolved in 95 per cent alcohol. The addition of a hot 3 per cent solution of lead acetate formed the corresponding lead salts. Those of the unsaturated group remained in solution whereas the others precipitated on standing in the ice chest overnight. This fact made it possible to separate the saturated from the unsaturated fatty acids. After the addition of dilute hydrochloric acid (1:3), the free acids thus liberated were extracted with petroleum ether, the solvent was removed by distillation, and the acids were weighed. The liquid fraction was next neutralized with alcoholic potassium hydroxide, the solvent removed by evaporation, and the soaps were dissolved in water. The corresponding barium salts were prepared by adding an aqueous solution of barium chloride, drop by drop, until a sudden coagulation of the precipitated soaps indicated the presence of a small excess of the precipitating reagent. The coagulum was removed by filtration and heated in a boiling water bath for half an hour in order to remove the major portion of the occluded water. The residual water was removed by means of an air current and the dry soaps were dissolved in hot benzene containing 5 per cent alcohol. On cooling and standing in the ice box for 24 hours, a gelatinuous precipitate of the barium soaps of oleic and traces of those of the saturated acids separated out. According to Farnsteiner (5), the filtrate from these soaps should contain the barium salts of the more highly unsaturated acids with but traces of barium oleate. A portion of the fraction was used for an iodine determination and the remainder reserved for further study. The acids of the solid fraction were dissolved in alcohol and exactly neutralized with alcoholic potassium hydroxide. After removal of the solvent, the dry potassium soaps were dissolved in 400 cc. of water and precipitated with a small excess of lithium acetate. The precipitated salts were filtered off and the filtrate was concentrated to half its original volume. On cooling, the lithium salt of lauric acid should separate out. The other lithium salts were decomposed with dilute hydrochloric acid (1:3) and the liberated acids extracted with petroleum ether. The potassium salts of these acids were then prepared in the manner described above, and dissolved in water. The addition of an excess of magnesium sulfate to this solution precipitated out the magnesium soaps. These were filtered off and digested with 50 per cent alcohol at 60° for ½ hour. The mixture was filtered hot and the filtrate cooled. The magnesium salt of myristic acid should separate out at this point.

## DISCUSSION.

From the data in Table I, in which is also given results of Sperry and Bloor (1) as well as those of Jaeckle (7), it is evident that in as far as the ratio of liquid to solid fatty acids is concerned, this tissue fat is like fecal fat. The sum of these two fractions in no case

XUM

accounts for all of the total fatty acid fraction. Sperry and Bloor (1) report a similar deficiency in their investigation. Like Sperry and Bloor, the writer observed the formation of an alcohol-insoluble residue during the purification of the lead salts. No attempt at the identification of the unknown was made, but the mere fact that it was consistently formed should account for the failure to obtain results totaling 100 per cent.

A separate determination of cholesterol by the digitonin method showed the presence of but 0.24 per cent of the sterol. Evidently there are other non-saponifiable substances present in human fat. In this connection, the writer wishes to call attention to some other work in the course of investigation which has shown the presence

TABLE I.

*Percentage of Solid Fatty Acids, Liquid Fatty Acids, and Non-Saponifiable Matter in the Subcutaneous Fat of Man.*

| Fat analyzed. | Non-saponifiable matter. | Total fatty acids. | Unsaturated fatty acids. | Saturated fatty acids. |
|---|---|---|---|---|
| *gm.* | *per cent* | *per cent* | *per cent* | *per cent* |
| 5.40 | 0.35 | 92.6 | 61 | 26 |
| 5.10 | 0.38 | 94.1 | 64 | 23 |
| 9.99 | 0.32 | 94.9 | 62 | 30 |
| 10.24 | 0.37 | 94.6 | 63 | 29 |
| 9.20 | 0.43 | 96.8 | 68 | 25 |
| Average..... | 0.37 | 94.6 | 63.6 | 26.6 |
| Feces (1) (dog)..... | | | 65 | 27 |
| Human (7)..... | | | 69.6 | 26 |

of 24 per cent of cholesterol in the fat of the outer layers of human skin. The total lipoids in the dry tissue were found to be 1.47 per cent. Calculated on this basis, there is 0.35 per cent of the sterol in the dry skin.

In some respects, the writer's analyses compare well with those reported by others for human fat. A study by Mitchell (8) shows an iodine number of 62 and a saponification number of 195. The values obtained by the writer were 65 and 196, respectively. Jaeckle's (7) results on the distribution of saturated and unsaturated fatty acids in the fat of obese individuals compare well with those found in this investigation. But Jaeckle differs from the writer in concluding that, of the unsaturated acids, oleic alone is present in human fat. The writer is at a loss to reconcile his own

XUM

results with this conclusion. As shown in Table II each sample analyzed yielded a fraction of barium salts soluble in cold benzene. It is true Lewkowitsch (9) found that some barium oleate invariably passed into the benzene filtrate and this observation has recently been confirmed by Lapworth, Pearson, and Mottram (10), but the fact that the iodine number of the acids in this fraction was invariably higher than that of oleic justifies the writer's subsequent conclusion that unsaturated acids other than oleic acid must be present in the abdominal fat of man. That this contention is warranted is evidenced by the fact that the addition of a drop of bromine to the ether solution of acids liberated from the benzene-soluble fraction invariably resulted in the formation of a

TABLE II.

*Amount of Fatty Acids Forming Barium Soaps Soluble in Cold Benzene.*

| Fat analyzed. | Fatty acids liberated from benzene-soluble barium soaps. | Iodine number of fatty acids liberated from benzene-soluble soaps. |
|---|---|---|
| *gm.* | *gm.* | |
| 5.40 | 1.080 | |
| 5.10 | 0.816 | |
| 9.99 | 1.630 | 115 |
| 10.24 | 1.740 | 110 |
| 9.20 | 2.110 | |
| 9.40 | 1.740 | 117 |
| 9.45 | 1.800 | 109 |
| 18.42 | 2.920 | 122 |

yellow precipitate, and when the solvent was removed and petroleum ether substituted for it, another yellow precipitate was formed. A characteristic property of fatty acids with more than two double bonds is the formation of brom addition compounds that are insoluble in ether. Linoleic acid, an acid with but two double bonds, and oleic acid also, add bromine. The compounds formed, however, are soluble in absolute ether and can thus be differentiated from the bromides prepared from the more highly unsaturated acids. The tetrabrom compounds of the linoleic series and dibrom compounds of the oleic series can be distinguished from each other for the reason that the former are insoluble in petroleum ether whereas the latter are not. The formation of

XUM

these insoluble bromides points to the presence of acids with at least two and three double bonds.

The amount of these acids in the samples analyzed was too small for detailed study. Larger amounts were therefore prepared by the fractionation of the methyl esters of the acids. The results of this fractionation are given in Table III. It is evident that the major portions of these esters (384 gm.) distilled between 193° and 197°C. The mere addition of a drop of liquid bromine to a small portion of each of the three high boiling fractions at once precipitated a yellow compound. Smaller amounts were also obtained from Fraction 6. The three high boiling fractions (Fractions 7, 8, and 9) were consequently combined, saponified

TABLE III.

*Fractions Obtained in the Distillation of the Methyl Esters of Human Fat.*

| Fraction No. | Boiling point at 7 mm. pressure. | Weight of fraction. |
|---|---|---|
| | °C. | gm. |
| 1 | 130–150 | 8 |
| 2 | 150–160 | 18 |
| 3 | 157–160 | 13 |
| 4 | 166–170 | 21 |
| 5 | 170–190 | 52 |
| 6 | 193–197 | 384 |
| 7 | 195–220 | 9 |
| 8 | 197–240 | 8 |
| 9 | 240–310 | 5 |

with alcoholic potassium hydroxide, and the fatty acids removed from the saponified mixture by acidifying with dilute hydrochloric acid and extraction with petroleum ether. This solvent was removed by distillation and the oily residue dissolved in 4 volumes of absolute ether. The brom addition compounds were then synthesized by adding an excess of liquid bromine. 3.5 gm. of the derivatives thus obtained were digested with boiling benzene for half an hour. The major portion remained undissolved while a small fraction (10 per cent of the total) passed into the hot filtrate from which a light colored crystalline compound separated on cooling. These facts suggest the presence of acids with three and four double bonds with a predominance of the latter for the reason that the hexabromide is soluble in hot benzene and the

XUM

octabromide is not. Bromine determinations of these compounds confirm this view. The benzene-insoluble compound contained 67 per cent of the halogen as compared with 61 per cent for the soluble material. These analyses check well with the theoretical amounts of bromine in the brom addition compounds of arachidonic and linolenic acids which are 67.6 and 63, respectively. An attempt was made to recover the arachidonic acid from its bromide by a method recently described by Wesson (11) who dissolved the addition compound in alcohol and removed the halogen therefrom by digestion with copper-coated zinc. As a result of this procedure a fatty acid with a distinct fishy odor was isolated from this mixture. Such an odor is characteristic of highly unsaturated fatty acids. The iodine number of this acid proved to be 288, which is lower than the theoretical number for arachidonic acid. This value is, nevertheless, higher than the theoretical figure for linolenic acid which is but 274. The fact that the purest preparation of linolenic acid described absorbed only 245 per cent of the halogen (12) is in accordance with the failure of the acid studied here to absorb its theoretical amounts of bromine.

The presence of acids with three and four double bonds has thus been established. That this is the case follows from the fact that human fat gives two different ether-insoluble bromides, the one being soluble in hot benzene, the other soluble therein. The percentages of halogen in these bromides check well with the theoretical values for acids with three and four double bonds. Furthermore, the iodine number of the predominating acid, arachidonic, agrees well with the actual value for this acid.

That an acid with two double bonds is likewise present is also true. The proof for its presence was the following. The ether in the filtrate from the insoluble bromides was evaporated off at room temperature and the light colored oil dissolved in an equal volume of boiling petroleum ether. On cooling and letting stand in the ice chest for 48 hours a light colored precipitate formed. These crystals were separated from the mother liquor by centrifuging and then dissolved in the minimum amount of absolute ether. A small amount of dark colored residue was filtered off, the solvent once more removed by evaporation at room temperature, and the oil dissolved in boiling petroleum ether. An almost white crystalline product separated out on cooling. The percent-

XUM

age of bromine therein was found to be 53, a value exactly checking that given for the tetrabromide of linoleic acid This fact, as well as the solubilities of the compound, is proof of the occurrence of linoleic acid in human fat. 1.2 per cent of this bromide was isolated from the fat. This corresponds to approximately 0.5 per cent for the free fatty acid. The writer is of the opinion that this figure is not a true index of the amount of linoleic acid in the tissue studied for the reason that available evidence (13) has shown that two addition products are formed when bromine is added to the petroleum ether solution of linoleic acid. One of these is a crystalline product and no doubt corresponds to the product isolated in this investigation. The other, however, is a liquid. Thus, only a part of the linoleic acid is determined with the method employed by the writer. He is not prepared to state how nearly quantitative the methods for the other unsaturated fatty acids are. Only 0.9 per cent of the octabromide and but 0.1 per cent of the hexabromide were obtained. The fact that approximately 17 per cent of the total fatty acids formed barium salts soluble in benzene would seem to indicate the presence of *large* amounts of fatty acids other than oleic. The iodine number of the acids in this fraction, being only 120, was too low to warrant such a conclusion. The writer must therefore concur with the conclusions of Lewkowitsch, and Lapworth, Pearson, and Mottram that the method is not a quantitative one. Results reported by Sperry and Bloor (1) seem to bear out this view. These investigators reported from 30 to 36 per cent of bromine in the ethereal filtrate from the ether-insoluble bromides obtained from fatty acids of the feces. If the soluble fraction had consisted of linoleic acid alone, then 53 per cent of bromine should have been absorbed.

The results secured from the attempt to isolate some of the constituents of the saturated fraction need little comment. The merest trace of an acid which might be lauric acid was obtained. Sufficient amounts for identification not being available, no conclusion was reached from the mere *suggestion* of its presence. It is possible that as much as 1 per cent of myristic acid is present in the hydrolyzed fat. The evidence, though not conclusive, is more convincing than that obtained for lauric acid, for though the melting point of an acid isolated by the alcohol extraction of the magnesium salts compares well with that given for myristic acid

and though the solubility of the magnesium salt of this acid differs from that of palmitic and stearic and is similar to that of myristic acid, the molecular weight as determined by titration is half-way between the molecular weights of palmitic and myristic acids which are 256 and 228, respectively. The result obtained was 239. It is true that the isolated acid was slightly pigmented, and this may perhaps account for the incorrect value for the molecular weight since a high value will be obtained when acids are thus contaminated. The melting point of this acid was 54° as compared with 53.8° for pure myristic acid. The fact that Jaeckle (7) has carefully studied the distribution of palmitic and stearic acids in fat similar to that studied in this investigation made it seem needless to repeat his work. The results reported by him were approximately 20 per cent palmitic and 5 per cent stearic acid.

The writer would like to take this opportunity to comment upon the method of fractional distillation of the methyl esters as a means for the quantitative separation of the fatty acids. The method does not give clean-cut fractions and is useful only in the isolation of certain fatty acids by a subsequent treatment of the fractions. This is suggested by the fact that Fractions 7, 8, and 9 which weighed 22 gm. yielded only 3.5 gm. of the brom addition compounds. Furthermore, the fact that the iodine number of the acids liberated from Fraction 6 was but 80 in spite of the fact that insoluble bromides were isolated from it, points to the presence of not only highly unsaturated esters but also of esters of the saturated acids. Upon redistillation of the fraction, 53 gm. of a distillate boiling at 197°C. were obtained, which, when analyzed, differed but little in composition from the whole fraction.

More data on the composition of depot fat are desirable, especially in view of the statement by Bloor (14) that it is rare to find in the stored, and, therefore, inactive, fat of warm blooded animals any acid less saturated than oleic. The writer proposes to study this question further.

## SUMMARY.

1. The ratio of liquid to solid fatty acids of the subcutaneous fat from the abdomen of man is similar to that reported by others for the fat of the blood and feces.

XUM

2. The presence of fatty acids with two, three, and four double bonds has been demonstrated for this depot fat. Of these linoleic acid predominates, 0.5 per cent having been isolated. The writer is of the opinion that more of this acid than this figure would indicate is present for the reason that, by the method employed, all of the acid is not precipitated as the crystalline tetrabromide. Only 0.33 per cent of the acid containing four double bonds and but 0.03 per cent of the triply unsaturated acid were found. To some extent at least, the distribution of the unsaturated acid of human abdominal fat is similar to that reported by others for the blood lipoids.

3. It is likely that myristic acid is present to the extent of about 1 per cent.

4. Evidence which suggested the presence of traces of lauric acid was secured.

5. 0.24 per cent of cholesterol as determined by the digitonin method was found.

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XUM

## INDEX TO AUTHORS.


A

**Adolph, Edward F.** Electrostatic forces in the diffusion of water through collodion membranes between solutions of mixed electrolytes, 339

B

**Baudisch, Oskar,** and **Davidson, David.** The mechanism of oxidation of thymine. 4, 5-Dihydroxyhydrothymine (thymine glycol), 233

— and **Welo, L. A.** On the aging of ferrous hydroxide and ferrous carbonate, 753

— and —. On the aging of natural mineral waters, 771

—. See DAVIDSON and BAUDISCH, 619

**Baumann, Emil J.,** and **Hunt, Louise.** On the relation of thyroid secretion to specific dynamic action, 709

**Becker, J. Ernestine.** See MCCOLLUM, SIMMONDS, and BECKER, 161

**Bell, Raymond W.** The effect of heat on the solubility of the calcium and phosphorus compounds in milk, 391

**Benedict, Stanley R.** The determination of blood sugar, 207

—. The determination of uric acid in the blood, 215

**Bezssonoff, Nikolaï.** The Jendrassik reaction for water-soluble B. Concerning Victor E. Levine's critical study, 589

**Biilmann, Einar.** See CULLEN and BIILMANN, 727

**Bills, Charles E.** The resistance of the antirachitic substance in cod liver oil to reagents, 1

**Black, Archie.** See STEENBOCK and BLACK, 263

**Bolliger, A.,** and **Hartmann, F. W.** Observations on blood phosphates as related to carbohydrate metabolism, 91

**Brand, Erwin.** See SANDBERG and BRAND, 59

**Brunquist, E. H.** See KOEHLER, BRUNQUIST, and LOEVENHART, 313

C

**Carrick, Carey W.** See HAUGE and CARRICK, 111

**Chown, H. Bruce.** See HOLT, LA MER, and CHOWN, 509, 567

**Clark, E. P.** See COLLIP and CLARK, 485

**Collens, William S.** The blood supply to the pancreas with some perfusion studies, 461

**Collins, Arnold M.** See JACOBS and COLLINS, 383

**Collip, J. B.,** and **Clark, E. P.** Further studies on the physi-




XUM

ological action of a parathyroid hormone, 485
**Cook, Kenneth G.** See ROSE and COOK, 325
**Cori, Carl F.,** and **Cori, Gerty T.** The carbohydrate metabolism of tumors. I. The free sugar, lactic acid, and glycogen content of malignant tumors, 11
**Cori, Gerty T.** See CORI and CORI, 11
**Csonka, Frank A.** See JONES and CSONKA, 673
**Cullen, Glenn E.,** and **Biilmann, Einar.** The use of the quinhydrone electrode for hydrion concentration determination on serum, 727
—. See DRUCKER and CULLEN, 221

D

**Davidson, David,** and **Baudisch, Oskar.** The oxidation of isobarbituric acid. A new class of indigoïds, 619
—. See BAUDISCH and DAVIDSON, 233
**Drucker, Paul,** and **Cullen, Glenn E.** A simple method for obtaining cutaneous (capillary) blood from infants and adults for colorimetric pH determination, 221
**Dunn, Edith R.** See VOEGTLIN, THOMPSON, and DUNN, 639

E

**Eckstein, H. C.** The fatty acids in the subcutaneous fat of man, 797
**Evenden, James.** See WIERZUCHOWSKI and LING, 697

F

**Fred, E. B.** See STILES, PETERSON, and FRED, 643

G

**Gersdorff, C. E. F.** See JONES and GERSDORFF, 241
—. See JONES, GERSDORFF, and MOELLER, 655
**Gittleman, I.** See HOLT, LA MER, and CHOWN, 567
**Goldschmidt, Samuel,** and **Light, Arthur B.** A method of obtaining from veins blood similar to arterial blood in gaseous content, 53
**Graham, Viola A.** See SUMNER and GRAHAM, 257
**Griffith, Wendell H.** A note on the influence of the diet on the synthesis of hippuric acid, 401
**Grollman, Arthur.** The combination of phenel red and proteins, 141
**Guerrant, Nollie B.** See HOGAN, GUERRANT, and KEMPSTER, 113

H

**Hammett, Frederick S.** A biochemical study of bone growth. I. Changes in the ash, organic matter, and water during growth (*Mus norvegicus albinus*), 409
—. II. Changes in the calcium, magnesium, and phosphorus of bone during growth, 685
—. III. Changes in the composition of the ash during growth, 693
**Harington, Charles Robert.** Synthesis of 3,4,5-triiodo-

phenylpyrrolidone carboxylic acid, a possible isomer of thyroxin, 29
**Hartman, F. W.** See BOLLIGER and HARTMAN, 91
**Hauge, Sigfred M.,** and **Carrick, Carey W.** The antiscorbutic properties of eggs, 111
**Helman, F. Dorothy.** See HESS and HELMAN, 781
**Hess, Alfred F.,** and **Helman, F. Dorothy.** The phosphatide and total phosphorus content of woman's and cow's milk, 781
— and **Weinstock, Mildred.** The antirachitic value of irradiated cholesterol and phytosterol. II. Further evidence of change in biological activity, 181
— and —. III. Evidence of chemical change as shown by absorption spectra, 193
**Hiller, Alma, Linder, G. C.,** and **Van Slyke, D. D.** The reducing substances of the blood, 625
**Hogan, Albert G., Guerrant, Nollie B.,** and **Kempster, Harry L.** Concerning the adequacy of synthetic diets for the growth of the chick, 113
**Holt, L. Emmett, Jr.** Studies in calcification. III. A quantitative study of the equilibria concerned with the calcification of bone, 579
——, **La Mer, Victor K.,** and **Chown, H. Bruce.** Studies in calcification. I. The solubility product of secondary and tertiary calcium phosphate under various conditions, 509
—, —, and —. II. Delayed equilibrium between the calcium phosphates and its biological significance, 567
**Hoppert, C. A.** See STEENBOCK and BLACK, 263
**Hunt, Louise.** See BAUMANN and HUNT, 709

J

**Jacobs, Walter A.** Saponins. III. The sapogenin occurring in *Sapindus saponaria* L. and *Sapindus mukorossi utilis* (Trabuti), 379
— and **Collins, Arnold M.** Strophanthin. VII. The double bond of strophanthidin, 383
**Jones, D. Breese,** and **Csonka, Frank A.** Proteins of the cottonseed, 673
— and **Gersdorff, C. E. F.** Proteins of wheat bran. II. Distribution of nitrogen, percentages of amino acids and of free amino nitrogen: a comparison of the bran proteins with the corresponding proteins of wheat endosperm and embryo, 241
—, —, and **Moeller, O.** Proteins of the bark of the common locust tree, *Robinia pseudacacia*. I. Enzymes associated with the proteins: the composition, properties, nitrogen distribution, and some of the amino acids of the albumin, 655

K

**Kempster, Harry L.** See HOGAN, GUERRANT, and KEMPSTER, 113

**Koehler, A. E., Brunquist, E. H.,** and **Loevenhart, A. S.** The production of acidosis by anoxemia, 313

— and **Reitzel, Raymond J.** The effect of pH on the oxygen consumption of tissues, 739

L

**La Mer, Victor K.** See HOLT, LA MER, and CHOWN, 509, 567

**Lawson, W. E.,** and **Scott, W. O.** An electrolytic modification of the Gutzeit method for the determination of arsenic in body tissues, 23

**Leiter, Louis.** The metabolism of imidazoles, 125

**Levene, P. A.,** and **Meyer, G. M.** Monoacetone galactose, 473

— and **Ulpts, R.** On condensation of monosaccharides by means of dilute mineral acid, 475

**Levine, Victor E.** The Jendrassik reaction for vitamin B with reference to the work of Bezssonoff and of Levine, 591

**Light, Arthur B.** See GOLDSCHMIDT and LIGHT. 53

**Linder, G. C.** See HILLER, LINDER, and VAN SLYKE, 625

**Ling, S. M.** See WIERZUCHOWSKI and LING, 697

**Locke, Arthur P.** See MAIN and LOCKE, 75

**Loevenhart, A. S.** See KOEHLER, BRUNQUIST, and LOEVENHART, 313

**Long, Esmond R.** See SEIBERT and LONG, 229

M

**MacLeod, F. L.** See SHERMAN and MACLEOD, 429

**Main, Edna Ruth,** and **Locke, Arthur P.** The determination of small amounts of protein nitrogen, 75

**McCollum, E. V., Simmonds, Nina,** and **Becker, J. Ernestine.** Further studies on the cause of ophthalmia in rats produced with diets containing vitamin A, 161

**Meyer, G. M.** See LEVENE and MEYER, 473

**Moeller, O.** See JONES, GERSDORFF, and MOELLER, 655

**Moritz, Alan R.** The effect of ultra-violet irradiation on the state of the serum calcium, 81

N

**Nelson, E. M.,** and **Steenbock, H.** Fat-soluble vitamins. XXIV. The non-precipitability of the antiophthalmic and antirachitic properties from cod liver oil by digitonin, 299

**Nelson, Mariana T.** See STEENBOCK and BLACK, 263

**du Noüy, P. Lecomte.** On the probable dimensions of the molecule and molecular weight of crystalline egg albumin, 595

P

**Peterson, W. H.** See STILES, PETERSON, and FRED, 643

R

**Read, Bernard E.** Chemical constituents of camel's urine, 615

**Reitzel, Raymond J.** See KOEHLER and REITZEL, 739

**Riising, Blanche M.** See STEENBOCK and BLACK, 263

**Rose, Anton R.** A micro method for determining nitrogen, 253

**Rose, William C.,** and **Cook, Kenneth G.** The relation of histidine and arginine to creatine and purine metabolism, 325

S

**Sackett, Guy E.** Modification of Bloor's method for the determination of cholesterol in whole blood or blood serum, 203

**Sandberg, Marta,** and **Brand, Erwin.** On papain lipase, 59

**Sando, Charles E.** Anthocyanin formation in *Helianthus annuus*, 71

**Scott, W. O.** See LAWSON and SCOTT, 23

**Seibert, Florence B.,** and **Long, Esmond R.** The interfering effect of glycerol on the biuret reaction, 229

**Sherman, H. C.,** and **MacLeod, F. L.** The calcium content of the body in relation to age, growth, and food, 429

**Simmonds, Nina.** See MCCOLLUM, SIMMONDS, and BECKER, 161

**Speakman, Horace B.** The biochemistry of acetone formation from sugars by *Bacillus acetoethylicum*, 41

**Steenbock, H.,** and **Black, Archie.** Fat-soluble vitamins. XXIII. The induction of growth-promoting and calcifying properties in fats and their unsaponifiable constituents by exposure to light, 263

—. See NELSON and STEENBOCK, 299

**Stiles, H. R., Peterson, W. H.,** and **Fred, E. B.** Fermentation products of certain mannitol-forming bacteria, 643

**Sumner, James B.,** and **Graham, Viola A.** The globulins of the jack bean (*Canavalia ensiformis*). II. The content of cystine, tyrosine, and tryptophane, 257

T

**Thompson, J. W.** See VOEGTLIN, THOMPSON, and DUNN, 639

U

**Ulpts, R.** See LEVENE and ULPTS, 475

V

**Van Slyke, D. D.** See HILLER, LINDER, and VAN SLYKE, 625

**Voegtlin, Carl, Thompson, J. W.,** and **Dunn, Edith R.** Hyperglycemia produced by glycerol, 639

W

**Weinstock, Mildred.** See Hess and Weinstock, 181, 193

**Welo, L. A.** See Baudisch and Welo, 753, 771

**Wierzuchowski, M.,** and **Ling, S. M.** Animal calorimetry. Twenty-ninth paper. On fat production in a young hog, 697

**Wu, Daisy Yen.** See Wu and Wu, 369

**Wu, Hsien,** and **Wu, Daisy Yen.** Nature of heat denaturation of proteins, 369

## INDEX TO SUBJECTS.


A

**Absorption spectra:**
Chemical change (HESS and WEINSTOCK) 193

**Acetone:**
Sugars by *Bacillus acetoethylicum* (SPEAKMAN) 41

**Acidosis:**
Anoxemia (KOEHLER, BRUNQUIST, and LOEVENHART) 313

**Albumin:**
Egg, crystalline, molecular weight (DU NOÜY) 595
Locust tree bark, amino acids (JONES, GERSDORFF, and MOELLER) 655
— — —, nitrogen distribution (JONES, GERSDORFF, and MOELLER) 655

**Amino acid(s):**
Albumin of locust tree bark (JONES, GERSDORFF, and MOELLER) 655
Proteins of wheat bran (JONES and GERSDORFF) 241

**Amino nitrogen:**
Proteins of wheat bran (JONES and GERSDORFF) 241

**Anoxemia:**
Acidosis (KOEHLER, BRUNQUIST, and LOEVENHART) 313

**Anthocyanin:**
*Helianthus annuus* (SANDO) 71

**Arginine:**
Creatine metabolism (ROSE and COOK) 325
Purine metabolism (ROSE and COOK) 325

**Arsenic:**
Body tissues, electrolytic modification of Gutzeit method (LAWSON and SCOTT) 23

**Artery:**
Blood, venous blood similar in gaseous content (GOLDSCHMIDT and LIGHT) 53

**Ash:**
Bone, calcium, during growth (HAMMETT) 693
—, during growth (HAMMETT) 409
—, magnesium, during growth (HAMMETT) 693
—, phosphorus, during growth (HAMMETT) 693

B

**Bacillus:**
*acetoethylicum*, acetone from sugars (SPEAKMAN) 41

**Bacterium:**
Mannitol-forming bacteria, fermentation products (STILES, PETERSON, and FRED) 643




XUM

**Biuret reaction:**
Glycerol (SEIBERT and LONG) 229
**Blood:**
Arterial, venous blood similar in gaseous content (GOLDSCHMIDT and LIGHT) 53
Capillary, for colorimetric pH determination (DRUCKER and CULLEN) 221
Cholesterol, modification of Bloor's method (SACKETT) 203
Cutaneous (capillary), for colorimetric pH determination (DRUCKER and CULLEN) 221
Pancreas (COLLENS) 461
Phosphates, related to carbohydrate metabolism (BOLLIGER and HARTMAN) 91
Reducing substances (HILLER, LINDER, and VAN SLYKE) 625
Serum, cholesterol, modification of Bloor's method (SACKETT) 203
Sugar (BENEDICT) 207
Uric acid (BENEDICT) 215
Venous, similar to arterial in gaseous content (GOLDSCHMIDT and LIGHT) 53
**Body:**
Calcium, relation to age, growth, and food (SHERMAN and MACLEOD) 429
**Bone:**
Ash, calcium, during growth (HAMMETT) 693
**Bone**—*continued:*
Ash, magnesium, during growth (HAMMETT) 693
—, phosphorus, during growth (HAMMETT) 693
Calcification, equilibria concerned (HOLT) 579
Calcium, during growth (HAMMETT) 685
Growth (HAMMETT) 409, 685, 693
Magnesium, during growth (HAMMETT) 685
Phosphorus, during growth (HAMMETT) 685

C

**Calcification:**
(HOLT) 579
(HOLT, LA MER, and CHOWN) 509, 567
Bone, equilibria concerned (HOLT) 579
**Calcium:**
Body, relation to age, growth, and food (SHERMAN and MACLEOD) 429
Bone ash during growth (HAMMETT) 693
— during growth (HAMMETT) 685
Milk, heat on solubility (BELL) 391
Phosphates, equilibrium between (HOLT, LA MER, and CHOWN) 567
— solubility product (HOLT, LA MER, and CHOWN) 509
Serum, ultra-violet irradiation (MORITZ) 81

**Calorimetry:**
Animal (WIERZUCHOWSKI and LING) 697
**Camel:**
Urine, chemical constituents (READ) 615
**Canavalia ensiformis:**
*See* Jack bean.
**Carbohydrate:**
Metabolism, relation of blood phosphates (BOLLIGER and HARTMAN) 91
— of tumors (CORI and CORI) 11
**Carboxylic acid:**
3,4,5-Triiodophenylpyrrolidone, synthesis (HARINGTON) 29
**Cholesterol:**
Blood, modification of Bloor's method (SACKETT) 203
— serum, modification of Bloor's method (SACKETT) 203
Irradiation, antirachitic value (HESS and WEINSTOCK) 181, 193
**Cod liver oil:**
Antiophthalmic properties, non-precipitability by digitonin (NELSON and STEENBOCK) 299
Antirachitic properties, non-precipitability by digitonin (NELSON and STEENBOCK) 299
— substance, resistance to reagents (BILLS) 1
**Collodion:**
Membranes, diffusion of water through (ADOLPH) 339
**Cottonseed:**
Proteins (JONES and CSONKA) 673
**Creatine:**
Metabolism, arginine (ROSE and COOK) 325
—, histidine (ROSE and COOK) 325
**Cystine:**
Globulins of jack bean (SUMNER and GRAHAM) 257

D

**Diet(s):**
Hippuric acid, synthesis (GRIFFITH) 401
Synthetic, and growth of chicks (HOGAN, GUERRANT, and KEMPSTER) 113
**Digitonin:**
Antiophthalmic properties, non-precipitability from cod liver oil (NELSON and STEENBOCK) 299
Antirachitic properties, non-precipitability from cod liver oil (NELSON and STEENBOCK) 299
**4,5-Dihydroxyhydrothymine:**
(BAUDISCH and DAVIDSON) 233

E

**Egg(s):**
Albumin, crystalline, molecular weight (DU NOÜY) 595
Antiscorbutic properties (HAUGE and CARRICK) 111
**Electrode:**
Quinhydrone, hydrion concentration determination on serum (CULLEN and BIILMANN) 727

XUM

**Electrolytes:**
Water diffusion through collodion membranes between solutions, electrostatic forces (ADOLPH) 339
**Enzymes:**
Proteins of locust tree (JONES, GERSDORFF, and MOELLER) 655
**Equilibrium:**
Bone calcification, equilibria concerned (HOLT) 579
Calcium phosphates (HOLT, LA MER, and CHOWN) 567

F

**Fat(s):**
Fatty acids in subcutaneous (ECKSTEIN) 797
Growth-promoting and calcifying properties (STEENBOCK and BLACK) 263
Hog (WIERZUCHOWSKI and LING) 697
-Soluble vitamins (NELSON and STEENBOCK) 299
— — (STEENBOCK and BLACK) 263
**Fatty acids:**
Fat, subcutaneous (ECKSTEIN) 797
**Fermentation:**
Products, mannitol-forming bacteria (STILES, PETERSON, and FRED) 643
**Ferrous carbonate:**
Aging (BAUDISCH and WELO) 753
**Ferrous hydroxide:**
Aging (BAUDISCH and WELO) 753

G

**Galactose:**
Monoacetone (LEVENE and MEYER) 473
**Gas:**
Blood, venous, similar to arterial blood in gaseous content (GOLDSCHMIDT and LIGHT) 53
**Globulins:**
Jack bean (SUMNER and GRAHAM) 257
**Glycerol:**
Biuret reaction (SEIBERT and LONG) 229
Hyperglycemia produced by (VOEGTLIN, THOMPSON, and DUNN) 639
**Glycogen:**
Tumors, malignant (CORI and CORI) 11
**Growth:**
Chick, and synthetic diets (HOGAN, GUERRANT, and KEMPSTER) 113
**Gutzeit method:**
Arsenic in body tissues, electrolytic modification (LAWSON and SCOTT) 23

H

**Heat:**
Calcium compounds in milk (BELL) 391
Phosphorus compounds in milk (BELL) 391
Proteins, heat denaturation (WU and WU) 369
**Helianthus annuus:**
Anthocyanin (SANDO) 71
**Hippuric acid:**
Diet (GRIFFITH) 401

XUM

**Histidine:**
Creatine metabolism (Rose and Cook) 325
Purine metabolism (Rose and Cook) 325
**Hydrion:**
Concentration determination on serum, quinhydrone electrode (Cullen and Biilmann) 727
**Hydrogen ion:**
Concentration, oxygen consumption of tissues (Koehler and Reitzel) 739
Determination, colorimetric, cutaneous (capillary) blood (Drucker and Cullen) 221
**Hyperglycemia:**
Glycerol (Voegtlin, Thompson, and Dunn) 639

I

**Imidazoles:**
Metabolism (Leiter) 125
**Indigoïds:**
(Davidson and Baudisch) 619
**Irradiation:**
Cholesterol, antirachitic value (Hess and Weinstock) 181, 193
Phytosterol, antirachitic value (Hess and Weinstock) 181, 193
Serum calcium, ultra-violet irradiation (Moritz) 81
**Isobarbituric acid:**
Oxidation (Davidson and Baudisch) 619
**Isomer:**
Thyroxin (Harington) 29

J

**Jack bean:**
Globulins (Sumner and Graham) 257
**Jendrassik reaction:**
Vitamin B (Levine) 591
Water-soluble B (Bezssonoff) 589

L

**Lactic acid:**
Tumors, malignant (Cori and Cori) 11
**Light:**
Fats, unsaponifiable constituents, by exposure (Steenbock and Black) 263
**Lipase:**
Papain (Sandberg and Brand) 59
**Locust tree:**
Proteins of bark (Jones, Gersdorff, and Moeller) 655

M

**Magnesium:**
Bone ash during growth (Hammett) 693
— during growth (Hammett) 685
**Mannitol:**
-Forming bacteria, fermentation products (Stiles, Peterson, and Fred) 643
**Membranes:**
Collodion, diffusion of water through (Adolph) 339

XUM

**Metabolism:**
Carbohydrate, relation of blood phosphates (BOLLIGER and HARTMAN) 91
Creatine, arginine (ROSE and COOK) 325
—, histidine (ROSE and COOK) 325
Imidazoles (LEITER) 125
Purine, arginine (ROSE and COOK) 325
—, histidine (ROSE and COOK) 325
Tumors, carbohydrate metabolism (CORI and CORI) 11
**Milk:**
Calcium compounds, heat on solubility (BELL) 391
Phosphatide (HESS and HELMAN) 781
Phosphorus (HESS and HELMAN) 781
— compounds, heat on solubility (BELL) 391
**Mineral:**
Water, natural, aging (BAUDISCH and WELO) 771
**Mineral acid:**
Monosaccharides, condensation (LEVENE and ULPTS) 475
**Molecular:**
Weight, crystalline egg albumin (DU NOÜY) 595
**Molecule:**
Dimensions (DU NOÜY) 595
**Monoacetone:**
Galactose (LEVENE and MEYER) 473
**Monosaccharides:**
Condensation by dilute mineral acid (LEVENE and ULPTS) 475

N

**Nitrogen:**
Albumin of locust tree bark (JONES, GERSDORFF, and MOELLER) 655
Micro method (ROSE) 253
Protein (MAIN and LOCKE) 75
Proteins of wheat bran (JONES and GERSDORFF) 241

O

**Oil:**
Cod liver. *See* Cod liver oil.
**Ophthalmia:**
Cod liver oil, non-precipitability of antiophthalmic properties by digitonin (NELSON and STEENBOCK) 299
Vitamin A-containing diets (MCCOLLUM, SIMMONDS, and BECKER) 161
**Organic matter:**
Bone, during growth (HAMMETT) 409
**Oxygen:**
Tissues, pH (KOEHLER and REITZEL) 739

P

**Pancreas:**
Blood supply (COLLENS) 461
**Papain:**
Lipase (SANDBERG and BRAND) 59

**Parathyroid:**
Hormone, physiological action (COLLIP and CLARK) 485
**Perfusion:**
(COLLENS) 461
**Phenol red:**
Proteins (GROLLMAN) 141
**Phosphate(s):**
Blood, related to carbohydrate metabolism (BOLLIGER and HARTMAN) 91
Calcium, equilibrium between (HOLT, LA MER, and CHOWN) 567
—, solubility product of (HOLT, LA MER, and CHOWN) 509
**Phosphatide:**
Milk (HESS and HELMAN) 781
**Phosphorus:**
Bone ash during growth (HAMMETT) 693
— during growth (HAMMETT) 685
Milk (HESS and HELMAN) 781
—, heat on solubility (BELL) 391
**Phytosterol:**
Irradiation, antirachitic value (HESS and WEINSTOCK) 181, 193
**Protein(s):**
Bark of locust tree (JONES, GERSDORFF, and MOELLER) 655
Cottonseed (JONES and CSONKA) 673
Locust tree, enzymes associated with (JONES, GERSDORFF, and MOELLER) 655
Heat denaturation (WU and WU) 369
**Protein(s)**—*continued:*
Nitrogen (MAIN and LOCKE) 75
Phenol red (GROLLMAN) 141
Wheat bran (JONES and GERSDORFF) 241
**Purine:**
Metabolism, arginine (ROSE and COOK) 325
—, histidine (ROSE and COOK) 325

## Q

**Quinhydrone:**
Electrode, hydrion concentration determination on serum (CULLEN and BIILMANN) 727

## R

**Rachitis:**
*See* Rickets.
**Reducing substances:**
Blood (HILLER, LINDER, and VAN SLYKE) 625
**Rickets:**
Antirachitic value of irradiated cholesterol (HESS and WEINSTOCK) 181, 193
— — — irradiated phytosterol (HESS and WEINSTOCK) 181, 193
Cod liver oil, non-precipitability of antirachitic properties by digitonin (NELSON and STEENBOCK) 299
— — —, resistance of antirachitic substance in, to reagents (BILLS) 1
**Robinia pseudacacia:**
*See* Locust tree.

XUM

S

**Sapindus mukorossi utilis (Trabuti):**
Sapogenin (JACOBS) 379
**Sapindus saponaria L.:**
Sapogenin (JACOBS) 379
**Sapogenin:**
*Sapindus mukorossi utilis* (Trabuti) (JACOBS) 379
— *saponaria* L. (JACOBS) 379
**Saponification:**
Light, unsaponifiable constituents of fats on exposure (STEENBOCK and BLACK) 263
**Scurvy:**
Eggs, antiscorbutic properties (HAUGE and CARRICK) 111
**Serum:**
Blood, cholesterol, modification of Bloor's method (SACKETT) 203
Calcium, ultra-violet irradiation (MORITZ) 81
Hydrion concentration determination, quinhydrone electrode (CULLEN and BIILMANN) 727
**Specific dynamic action:**
Thyroid secretion, relation (BAUMANN and HUNT) 709
**Strophanthidin:**
Double bond (JACOBS and COLLINS) 383
**Strophanthin:**
(JACOBS and COLLINS) 383
**Sugar(s):**
Acetone from, by *Bacillus acetoethylicum* (SPEAKMAN) 41
Blood (BENEDICT) 207
Tumors, malignant, free sugar (CORI and CORI) 11

T

**Thymine:**
Oxidation (BAUDISCH and DAVIDSON) 233
**Thymine glycol:**
(BAUDISCH and DAVIDSON) 233
**Thyroid:**
Secretion, relation to specific dynamic action (BAUMANN and HUNT) 709
**Thyroxin:**
Isomer (HARINGTON) 29
**Tissues:**
Body, electrolytic modification of Gutzeit method for arsenic (LAWSON and SCOTT) 23
Oxygen consumption, pH (KOEHLER and REITZEL) 739
**3,4,5-Triiodophenylpyrrolidone carboxylic acid:**
Synthesis (HARINGTON) 29
**Tryptophane:**
Globulins of jack bean (SUMNER and GRAHAM) 257
**Tumors:**
Glycogen (CORI and CORI) 11
Lactic acid (CORI and CORI) 11

**Tumors**—*continued:*
Metabolism, carbohydrate (CORI and CORI) 11
Sugar, free (CORI and CORI) 11
**Tyrosine:**
Globulins of jack bean (SUMNER and GRAHAM) 257

U

**Uric acid:**
Blood (BENEDICT) 215
**Urine:**
Camel, chemical constituents (READ) 615

V

**Vein(s):**
Blood similar to arterial in gaseous content (GOLDSCHMIDT and LIGHT) 53
**Vitamin(s):**
Fat-soluble (NELSON and STEENBOCK) 299
— (STEENBOCK and BLACK) 263
**Vitamin A:**
Ophthalmia in rats produced with diets containing (MCCOLLUM, SIMMONDS, and BECKER) 161
**Vitamin B:**
Jendrassik reaction (BEZSSONOFF) 589
— — (LEVINE) 591

W

**Water:**
Bone, during growth (HAMMETT) 409
Diffusion through collodion membranes between solutions of mixed electrolytes, electrostatic forces (ADOLPH) 339
Mineral, natural, aging (BAUDISCH and WELO) 771
**Water-soluble B:**
Jendrassik reaction (BEZSSONOFF) 589
**Wheat:**
Bran, amino acids of proteins (JONES and GERSDORFF) 241
—, amino nitrogen of proteins (JONES and GERSDORFF) 241
—, nitrogen of proteins (JONES and GERSDORFF) 241
—, proteins (JONES and GERSDORFF) 241
—, —, comparison with proteins of wheat embryo (JONES and GERSDORFF) 241
—, —, — with proteins of wheat endosperm (JONES and GERSDORFF) 241
Embryo, proteins, comparison with proteins of wheat bran (JONES and GERSDORFF) 241
Endosperm, proteins, comparison with proteins of wheat bran (JONES and GERSDORFF) 241

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